DOCTOR OF PHILOSOPHY

Catalytic and non-catalytic mechanisms involved in Glutathione S-transferase Pi mediated cytoprotection

McGarry, David

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Declaration

I declare that this thesis is based on results obtained from investigations which I have personally carried out, and that the entire thesis is my own composition. Any work other than my own is clearly stated in the text and acknowledged with reference to any relevant investigators or contributors. This thesis has never been presented previously, in whole or in part, for the award of any higher degree. I have consulted all the references cited within the text of this thesis.

Signed …… Date …….....

I confirm that David McGarry has spent the equivalent of at least 9 terms in the Division of Cancer Research, Medical Research Institute, University of Dundee, and that he has fulfilled the conditions of the University of Dundee, thereby qualifying him to submit this thesis in application for the degree of Doctor of Philosophy.

Signed …… Date …….....

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Abbreviations

$(NH_4)_2SO_4$	Ammonium sulphate
15-d-PGJ2	15-deoxy-$\Delta^{12,14}$prostaglandin J2
17-AAG	17-N-Allylamino-17-demethoxygeldanamycin
ABC	ATP binding cassette
ALT	Alanine aminotransferase
AP-1	Activator protein-1
APAP	Acetaminophen
APC	Adenomous polyposis coli
ARE/EpRE	Antioxidant/Electrophile Response Element
ASK1	Apoptosis signal-regulating kinase 1
AST	Aspartate aminotransferase
ATF2	Activating transcription factor 2
ATP	Adenosine-5'-triphosphate
BAC	Bacterial artificial chromosome
BaP	Benzo[a]pyrene
BioGEE	Biotinylated glutathione ethyl ester
BSA	Bovine serum albumin

BSO	Buthionine sulfoximine
$CaCl_2$	Calcium Chloride
Cdk5	Cyclin dependent kinase-5
CDNB	1-chloro-2,4-dinitrobenzene
CREB	cAMP response element-binding protein
CYP	Cytochrome P450
DMBA	7,12-dimethylbenz anthracene
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNIC	Dinitrosyl-diglutathionyl iron complex
DNP	2,4-dinitrophenol
DNP-SG ATPase	Anion transporter dinitrophenol S-glutathione
dNTP	Deoxyribonucleotide triphosphate
DTNB	5,5’-dithio-bis (2-nitrobenzoic acid)
DTT	Dithiothreitol
E. Coli	Escherichia coli
EA	Ethacrynic acid
ECAR	Extracellular acidification rate

EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
eIF4E	Eukaryotic initiation factor 4E
EKLF	Erythroid Krüppel-like Factor
ERK	Extracellular signal-regulated kinases
ETF	Electron transfer flavoprotein
FANCC	Fanconi anemia group C protein
FBS	Fetal bovine serum
FKHD	Fork Head Protein
Flp	Flippase recombination enzyme
FMO	Flavin-containing monooxygenase
FRT	Flippase recognition target
FSG	Fish Skin Gelatin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Glutamate cysteine ligase
GFP	Green fluorescent protein
GGT	γ-glutamyltranspeptidase

GPE1	GSTP1 Enhancer 1
Grx	Glutaredoxin
GS	Glutathione synthetase
GSH	Glutathione
GSNO	S-nitrosoglutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
H/E	Hematoxylin and eosin
H_2DCFDA	Dichlorodihydrofluorescein Diacetate
HCL	Hydrochloric acid
HDAC	histone deactetylase
HO-1	Haem oxygenase-1
HPV-16 E7	Human Papillomavirus (HPV)-16 E7
Hsp90	Heat shock protein 90
IEF	Isoelectric focusing
IFNγ	Interferon-gamma
iNOS	inducible Nitric oxide synthase
IPG	Immobilized pH gradient

IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRES-ßGEO	Internal ribosome entry site -β-galactosidase reporter neomycin
JNK	c-Jun N-terminal kinase
KCl	Potassium Chloride
Keap1	Kelch-like ECH associating protein 1
KPE	Potassium phosphate buffer with EDTA
KRAS	Kirsten rat sarcoma viral oncogene
LB	Lysogeny broth
LDH	Lactate dehydrogenase
L-NAME	N-Nitro-L-arginine methyl ester hydrochloride
Maf	Musculo-aponeurotic fibrosarcoma
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
MAP Kinase	Mitogen-activated protein kinase
MAPKKK	MAP Kinase Kinase Kinase
MDM2	murine double minute 2
MEF	Mouse embryonic fibroblast
MEKK1	MAP/ERK Kinase Kinase 1

MgCl2	Magnesium Chloride
$MgSO_4$	Magnesium sulphate
MKNK2	MAP kinase interacting serine/threonine kinase 2
MnSOD	Manganese superoxide dismutase
MOZ	Monocytic leukaemia zinc-finger protein
MRP	Multidrug resistance protein
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
NAPQI	N-acetyl-*p*-benzoquinone imine
NEM	N-ethylmaleimide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NQO1	NAD(P)H: quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
OATP2	Organic anion transporting polypeptide 2
OCR	Oxygen consumption rate
PAHs	Polycyclic aromatic hydrocarbons

PAPS	3’-phosphoadenosine-5’-phosphosulfate
PBS	Phosphate buffered saline
PBST	PBS/Tween solution
PCR	Polymerase chain reaction
PI	Propidium Iodide
PKA/PKC	cAMP-dependent protein kinase A/C
PPARγ	Peroxisomal proliferator-activated receptor γ
PrdxVI	Peroxiredoxin VI
PSNO	S-nitrosothiol
RARE	Retinoic acid response element
RGS2	Regulator of G-protein Signalling 2
RNS	Reactive nitrogen species
ROS	Reactive oxidative species
SAM	S-Adenosyl-l-methionine
SDS	Sodium dodecyl sulfate
shRNA	short hairpin RNA
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription

tBHQ	Tert-butylhydroquinone
TBST	Tris buffered saline and Tween 20
TGM2	Transglutaminase 2
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAF2	Tumour necrosis factor receptor-associated factor 2

Contents

List of Figures

List of Tables

Abstract

Glutathione S-transferases (GSTs) are a multi-gene family of enzymes involved in the detoxification of a wide range of electrophilic compounds and are an integral component to antioxidant defence in the mammalian cell. Among GST, the Pi class (GSTP1) is the most prominent extrahepatic isoform in humans, and it is well documented that increased expression of this enzyme is present in human tumours and can contribute to anticancer drug resistance, involving many compounds which are not known substrates for GSTP1. In addition to xenobiotic metabolism, GSTP1 has a key and varied role in cell regulation, showing to be a modulator of a stress response pathway and has been implicated in mediating sulfhydryl homeostasis.

The present study aims to address functions of GSTP1 which are independent of its catalytic activity. Initial *in vivo* studies have identified that targeting a Tyr7Phe substitution in the murine *Gstp1* gene results in a GSTP1 protein which is devoid of catalytic activity. When challenged with a toxic dose of acetaminophen, these mice show a high degree of resistance to the hepatotoxic effects of this compound compared to their wild-type counterparts, in part, due to a partial recovery of hepatic glutathione levels. The ability of GSTP1 to mediate glutathione homeostasis was demonstrated *in vitro,* where silencing of GSTP1 in a colon carcinoma cell line demonstrated increased levels of glutathione disulphide and protein S-glutathionylation, a reversible post-translational modification involved in thiol protection. Subsequent analysis of GSTP1 silencing in this cell line led to the identification of potential novel pathways which may be mediated by GSTP1.This study examines the regulatory processes mediated by GSTP1 and aims to further our understanding of this protein in the aetiology of disease and toxicity.

1. Introduction

Since initial pioneering studies by Freidrich Woehler on the synthesis of urea and Alexandra Ure's observation on the conversion of benzoic acid to hippuric acid in humans in the mid-19th Century, the understanding of toxicology and drug metabolism has become pivotal in understanding how organisms are able to protect themselves against the diverse range of chemicals that they are exposed to. The ability of an organism to metabolise compounds is essential in survival, not only in avoiding toxicity through the detoxification of chemicals, but also in the breakdown of foods and nutrients for growth. Organisms are exposed to a variety of chemicals through a number of different routes. For example, oxygen which is necessary for cellular respiration is incredibly chemically reactive, often producing oxidative radicals as a reaction by-product which can lead to cytotoxicity. As such, a sophisticated system of detoxification is necessary in maintaining cellular homeostasis and mediating cytoprotection.

Despite over 150 years of study into drug metabolism, our understanding of the mechanisms involved in these processes remains incomplete. Despite their importance in biological processes from prokaryotes to eukaryotes, the composition and genetic variation of drug metabolising enzymes (DMEs) vary across species, ethnicities and individuals, which complicates the ability to predict the metabolism and activity of a given compound. To add further complexity, DMEs have been found to possess cellular functions independent of metabolism, adding layers of intricacy in understanding how cellular defence systems are integrated. The focus of this thesis will describe how one particular group of DMEs, the glutathione S-transferases, are involved in regulating cellular functions other than drug metabolism and begin to describe the importance of this enzyme in relation to toxicology and tumorigenesis.

1.1 Drug metabolism

Metabolism of xenobiotic compounds largely involves the breakdown or inactivation of a lipophilic substrate into a more water soluble and readily excretable by-product. Prof. RT Williams initially categorised xenobiotic metabolised in 2 distinct phases (Williams, 1972) known as Phase I and Phase II, although a third phase has since been added (Figure 1.1). Phase I reactions typically involve oxidation, reduction or hydrolytic reactions which leads to the functionalization of a substrate. Phase I reactions are catalysed primarily by enzymes from the cytochrome P450 (CYP) family of proteins, so called due to a peak at 450nm in the reduced carbon monoxide absorption spectrum. CYPs are a diverse family of hemoproteins which facilitate the metabolism of a wide range of exogenous compounds as well as regulation of a number of endogenous substrates such as retinoic acid (Otto et al., 2003), cholesterol (Henderson et al., 2003) and steroid hormones (Miller, 1988). CYPs act as an electron acceptor from cytochrome P450 oxidoreductase to catalyse the addition of oxygen into a substrate using NAPDH as a cofactor. The multiplicity of CYP family members demonstrates a remarkable diversity in their reaction chemistry, despite evolving from a common ancestral gene (Nebert et al., 1987). Reactive metabolites formed from this reaction are conjugated with an endogenous substrate through a Phase II reaction which typically results in the inactivation of the metabolite. The conjugated substrate can then be excreted from the body as a polar compound via drug transporters in what can be regarded as a Phase III reaction. Due to the often bulky nature of the conjugating group or acidity of the conjugate (as is the case for glutathione conjugation), Phase III reactions involve the efflux of a conjugated-metabolite via a drug transporter such as the ATP binding cassette (ABC) family of transporters. Drug transporters are ubiquitous throughout the body and form an integral component of the detoxification system in a number of organs, often found highly expressed in epithelial and endothelial compartments such as the blood brain barrier and the

blood-testes barrier. For the purpose of this introduction, this thesis will focus principally on Phase II metabolism although references to CYP mediated reactions and the role of drug transporters in toxicology are provided (Guengerich, 2008, Fletcher et al., 2010, Coon, 2005).

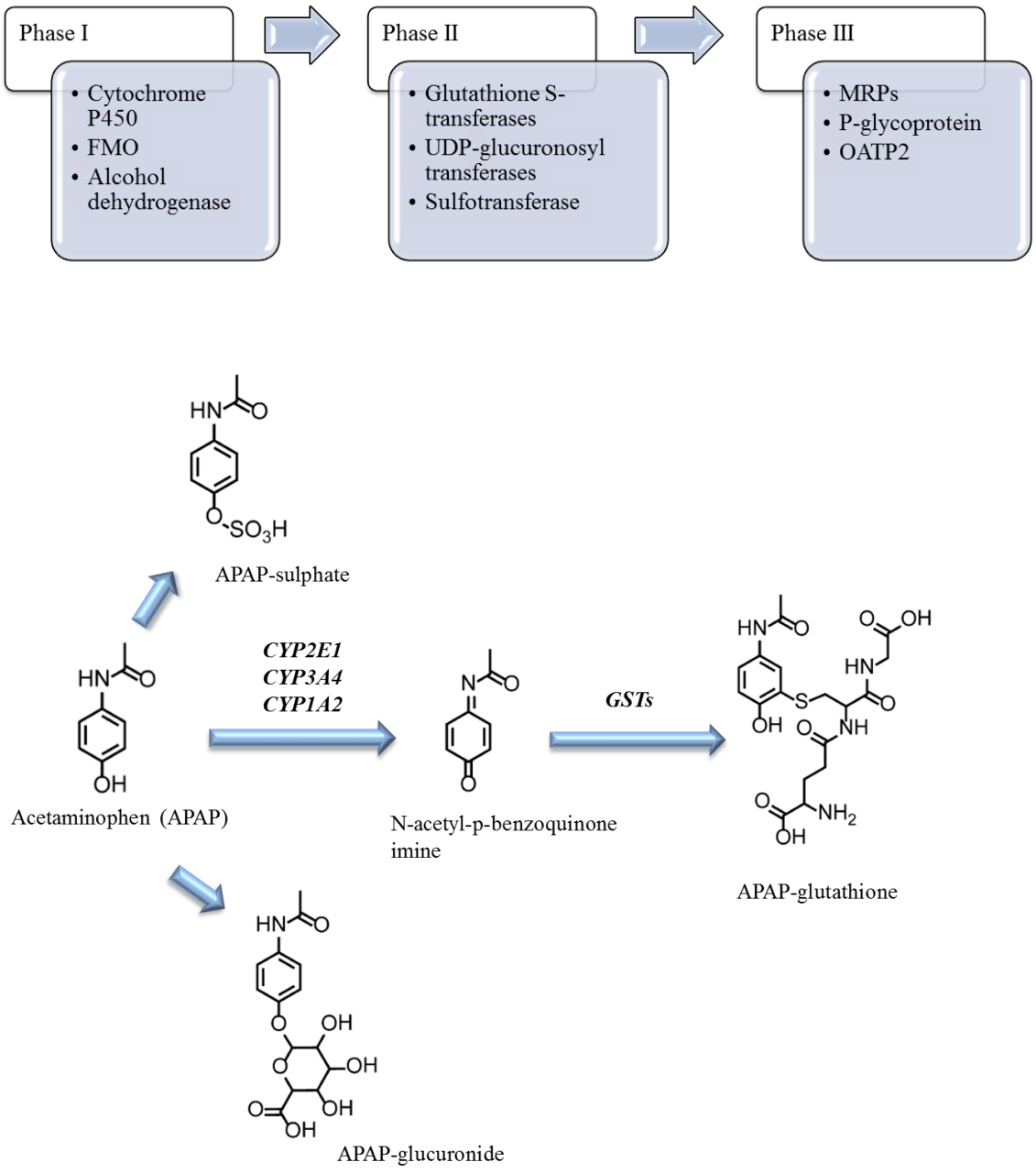


Figure 1.1. A schematic diagram demonstrating the phases of drug metabolism.

Xenobiotic compounds are typically metabolised in 3 distinct phases of metabolism highlighted in the top diagram. Enzymes typically involved in the catalysis of these reactions are described at each stage. An example

of drug metabolism is provided in the lower diagram; acetaminophen (APAP) can be conjugated directly to form glucuronide and sulphate derivatives or metabolised by cytochrome P450 enzymes into a highly reactive N-acetyl-p-benzoquinone imine metabolite. This can then be conjugated to glutathione spontaneously or catalytically by glutathione S-transferases (GSTs). FMO, flavin-containing monooxygenase; MRP, multidrug resistance protein; OATP2, organic anion transporting polypeptide 2.

1.2 Phase II metabolism

Phase II reactions involve the addition of a metabolite which utilises a high energy intermediate such as UDP-glucuronic acid, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), S-Adenosyl-l-methionine (SAM), acyl-Coenzyme A or utilises a reactive nucleophilic centre as is the case for glutathione conjugation. These endogenous substrates are catalysed through a number of enzymes and can facilitate the conjugation of a diverse set of reactive groups as highlighted in Table 1.

In general, Phase II reactions result in the detoxification of a substrate whereby the reactive group becomes highly polar allowing it to be readily excreted into the bile or urine. However, there are some instances where conjugation may enhance the toxicity of a compound. Short-chain alkyl halides and dichloromethane still possess electrophilic moieties after glutathione conjugation (Wheeler et al., 2001) while the glutathione-platinum conjugate produced after cisplatin metabolism is a substrate for γ-glutamyltranspeptidase and cysteine S-conjugate β-lyase in the proximal tubules, which results in the formation of reactive thiols (Hanigan et al., 2001).

Conjugation Reaction	Enzyme	Functional group	Enzyme localisation
Glucuronidation	UDP-glucuronosyltransferase	-OH -COOH $-NH_2$ -SH	Endoplasmic reticulum
Sulfation	Sulfotransferase	$-NH_2$ -OH	Cytosol Golgi apparatus
Methylation	Methyltransferase	-OH $-NH_2$ -SH	Cytosol Endoplasmic reticulum
Acetylation	N-acetyltransferase	$-NH_2$ -OH	Cytosol
Glutathione	Glutathione S-transferase	Epoxides Organic halides	Cytosol Peroxisomes Mitochondria Nucleus
Amino acid		-COOH	Mitochondria

Table 1. Phase II conjugation reactions and their enzymes.

Induction of Phase II enzymes can be mediated by a number of dietary compounds such as flavonoids (Han et al., 2012), isothiocyanates (Zhang et al., 1992), phenolic compounds such as butylated hydroxyanisole (McLellan et al., 1994, McLellan et al., 1992) and green tea polyphenols (Khan et al., 1992) which act through an electrophilic mediated stress response resulting in activation of the Antioxidant/Electrophile Response Element (ARE/EpRE) embedded in the promoter region of Phase II enzymes (Itoh et al., 1997). Typically, induction of Phase II enzymes occurs through the binding of a cap'n'collar basic-region leucine zipper protein, nuclear factor erythroid 2-related factor 2 (Nrf2) to the ARE which, in a heterodimer

complex with a musculo-aponeurotic fibrosarcoma (Maf) protein, act to recruit CREB binding proteins and p300 (Zhu and Fahl, 2001) to facilitate the transcription of Phase II enzymes along with other genes involved in cytoprotection such as haem oxygenase-1 (HO-1) (Alam et al., 1999) and glutamate cysteine ligase (GCL) (McWalter et al., 2004). Basally, Nrf2 binds to kelch-like ECH associating protein 1 (Keap1) in the cytoplasm (Itoh et al., 1999), which results in its ubiquitination through an interaction with the E3-based ligase, Cullin 3 (Kobayashi et al., 2004, McMahon et al., 2003). The ability of Keap1 to suppress Nrf2 activity is redox dependent. Keap1 contains 25 cysteine residues, nearly 1:3 of which are flanked by basic amino acid residues which reduces their pKa value, enhancing their reactivity (this form of reactivity is discussed further in Chapter 4) (Dinkova-Kostova et al., 2001). Interaction of these residues with a number of thiol reactive electrophiles (Itoh et al., 2003) and endogenous signalling molecules (McMahon et al., 2010) results in the inactivation of Keap1 and activation of Nrf2, where it can translocate to the nucleus and interact with the ARE. Therefore induction of Phase II enzymes can also occur through the production of thiol reactive metabolites indirectly produced as a consequence of cellular stress, such as the production of reactive oxidative species (ROS) and free radicals. This mechanism outlines an adaptive antioxidant system in which Phase II enzymes form a central component in the response to cellular stress in addition to their role in drug metabolism.

1.3 Glutathione S-transferases

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a multi-gene family of enzymes involved in the detoxification of a wide range of electrophilic compounds and are an integral component to antioxidant defence in the mammalian cell (Hayes and Pulford, 1995). Found in multiple cytosolic and membrane-bound forms, GSTs catalyse the conjugation of the ubiquitous tripeptide glutathione in its reduced state (GSH), to reactive xenobiotic and

endogenous substrates. GSTs were discovered in 1961 as an enzyme conjugating glutathione to halogenated aromatic compounds (Combes and Stakelum, 1961, Booth et al., 1961), but were soon found to have activity with a wide range of dissimilar substrates (Pabst et al., 1973). In addition to xenobiotic metabolism, GSTs have a key and varied role in cell regulation, including the biosynthesis of many endogenous substrates, response mechanisms in oxidative stress and modulation of several signalling pathways. Furthermore, there is evidence to suggest that polymorphisms of GSTs are implicated in the aetiology of many human diseases such as asthma (A. Sükrü Aynacioglu, 2004, Zhou et al., 2008), liver disease (Alexandra Henrion-Caude, 2002), Parkinsonism (Yoritaka et al., 1996) Alzheimer's (Pinhel et al., 2008), cancer (Huang et al., 2009, Mitrunen et al., 2001, Funke et al., 2010, Stoehlmacher et al., 2002) and that overexpression of some GST isoenzymes may make a significant contribution to the development of tumorigenesis and anticancer drug resistance (Goto et al., 1999, Dang et al., 2005, Funke et al., 2010, Black and Wolf, 1991).

1.3.1 GST nomenclature and structure

There are 3 mammalian classes of GST which share >50% amino acid sequence identities; cytosolic (canonical), mitochondrial (Kappa class) and membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Atkinson and Babbitt, 2009). A fourth class of GST has been identified in bacteria as a 16kDa polypeptide metalloenzyme known as FosA which catalyzes the addition of glutathione to the antibiotic fosfomycin (Arca et al., 1990). A nomenclature system proposed by Mannervik et al. provides a detailed outline for GST designation (Mannervik et al., 1992) and has since been revised (Mannervik et al., 2005). Enzyme classes are named in Greek and abbreviated with the corresponding Roman letter. For example, the class GST Alpha would be abbreviated as GSTA. Cytosolic GSTs are dimeric proteins and subunit components are represented by Arabic numerals. GST genes are

italicised and allelic variants are represented by lower case letters. If more than one species is described, it is prefixed with a lower case letter denoting the species. For example, human GSTP1 is described as hGSTP1 and mouse as mGSTP1. An overview of the different classes of human GSTs along with details of their substrate reactivity is provided in Table 2.

Of the GST family, the cytosolic class are the most abundant and widely-studied group and are categorised according to their amino acid sequence similarities (Mannervik and Danielson, 1988, Hayes et al., 2005). To date there are 7 classes of mammalian cytosolic GSTs; Alpha, Mu, Pi (Mannervik et al., 1985b), Sigma (Meyer and Thomas, 1995), Theta (Meyer et al., 1991), Zeta (Board et al., 1997) and Omega (Board et al., 2000), although other forms have been found in different species (Sheehan et al., 2001). Cytosolic GSTs are composed of dimers of approximately 25kDa subunits. Each subunit contains two distinct domains; a glutathione-binding 'G-site' and a hydrophobic ligand binding 'H-site' (Reinemer et al., 1991, Reinemer et al., 1992, Wilce et al., 1995, Sinning et al., 1993). The G-site is found within the N-terminal domain and is highly conserved throughout the classes, containing a serine or tyrosine molecule to stabilise glutathione binding (Reinemer et al., 1991, Wilce et al., 1995). The fold of the N-terminal domain contains a βαβαββα motif which is similar to that of the thioredoxin family of enzymes (Figure 1.2) and is important in recognising the γ-glutamyl peptide in glutathione binding (Robinson et al., 2004, Atkinson and Babbitt, 2009). The C-terminus, composed of mainly α-helices, contains the H-site which is not conserved between classes and can accommodate a wide range of electrophilic compounds containing a carbon, nitrogen or sulphur functional group (Hayes and Pulford, 1995). As a result, few ligands can be used to identify individual classes, although the structural differences in the H-site domain between GST classes does allow for some substrates to be preferentially bound than others. For example, GSTA4 has strong activity

towards products of lipid peroxidation such as 4-hydroxynonenal (Zimniak et al., 1992), while GSTP1 has preferential activity towards ethacrynic acid (Phillips and Mantle, 1993). The nucleophilic substitution of 1-chloro-2,4-dinitrobenzene (CDNB) to S-(2,4-dinitrophenyl)glutathione can be commonly catalysed by most GSTs as it is relatively small and sterically compliant compared to the H-site and can therefore be used as a universal substrate for studying GST activity (Habig et al., 1974).

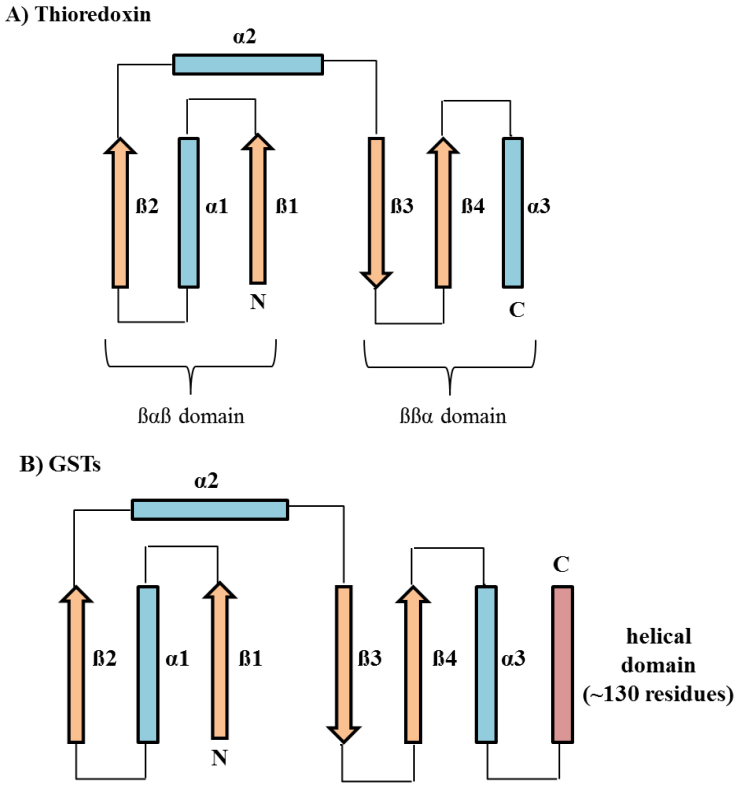


Figure 1.2. Schematic topology diagram demonstrating the evolution of the N-terminal fold of GSTs from thioredoxin.

Topology diagram showing the N-terminal fold of thioredoxin **(A)** and canonical GSTs **(B)**. GSTK1 differs from the canonical fold of GSTs in that its helical domain is inserted in the α2 connecting helix. α-helices are presented in blue and ß-helices in orange. Adapted from Robinson et al 2004.

1.3.2 Role in drug metabolism and chemical detoxification

The predominant action of GSTs is in the detoxification of xenobiotic or endogenous compounds. GSTs catalyse the conjugation of reduced glutathione to many electrophilic moieties as a glutathionyl thiolate anion (GS^-). The binding of GSH to GST results in a lowering of the pKa of GSH from 9.2 to 6.5 when bound in the active site. This results in the stabilisation of GS^- which can then spontaneously react with a diverse range of electrophilic compounds (Boyland and Chasseaud, 1969, Chasseaud, 1979). A foreign compound can be conjugated to GSH catalytically by one of two main metabolic routes; addition and nucleophilic substitutions. Addition reactions involve the transfer of a proton from the thiol group to the target compound and are common to most reactions catalysed by GSTs such as those of epoxides, isothiocyanates, quinones and alkenes (Berhane et al., 1994, Robertson et al., 1986, van Ommen et al., 1991, Chasseaud, 1979, Keen et al., 1976). Substitution reactions involve the functional group of a compound being replaced with the GSH thiolate such as halides (Saavedra et al., 2001). The resulting glutathione-conjugate can be transported from the cell into the bile through a variety of transport systems; the ABC family multidrug resistance-associated proteins 1 and 2 (MRP1 and MRP2) (Loe et al., 1998); the anion transporter dinitrophenol S-glutathione (DNP-SG ATPase) (Saxena et al., 1992) and a Ral binding protein (RLIP76) which has similar structural and functional properties to DNP-SG ATPase (Awasthi et al., 2000). Awasthi et al have proposed that the two latter systems contribute to the majority of glutathione-conjugate transport in the mammalian cell (Awasthi et al., 2005). After transportation from the cell, the conjugate is cleaved in the liver or kidney by γ-glutamyltranspeptidase and dipeptidases to yield a cysteine conjugate. This substrate may then undergo a series of N-acetylation reactions to produce N-acetylcysteine conjugates or mercapturic acid (Pabst et al., 1973, Keen and Jakoby, 1978).

Enzyme	**Gene**	**Chromosome location**	**Class**	**Residue to stabilise GS^- thiolate anion**	**Enzyme activity/reaction**
GSTA1	*GSTA1*	6p12	Cytosolic	Tyr9	CDNB; Δ^5-ADD, BCDE, BPDE, Busulfan, Chlorambucil, DBADE, DBPDE, BPhDE, NBD-Cl; N-a-PhIP; PGE_2; PGF_{2a} synthase
GSTA2-2	*GSTA2*	6p12	Cytosolic	Tyr9	CuOOH, DBPDE, CDNB, NBD-Cl, CuOOH, PGD_2 and PGF_{2a} synthase
GSTA3-3	*GSTA3*	6p12	Cytosolic	Tyr9	Δ^5-ADD, Δ^5-pregnene-3,20-dione, DBPDE
GSTA4-4	*GSTA4*	6p12	Cytosolic	Tyr9	COMC-6, EA, 4-hydroxynonenal, 4-hydroxydecenal, CDNB
GSTA5-5	*GSTA5*	6p12	Cytosolic	Tyr9	CDNB, 4-hydroxynonenal, *trans*-nonenal, acrolein
GSTM1-1	*GSTM1*	1p13.3	Cytosolic	Tyr6	*trans*-4-phenyl-3-buten-2-one, BPDE, CDE, DBADE, *trans*-stilbene oxide, styrene-7,8-oxide, CDNB, AFB1-epoxide
GSTM2-2	*GSTM2*	1p13.3	Cytosolic	Tyr6	COMC-6, 1,2-

					dichloro-4-nitrobenzene, aminochrome, dopa *O*-quinone, $PGH_2 \rightarrow PGE_2$, CDNB,
GSTM3-3	*GSTM3*	1p13.3	Cytosolic	Tyr6	BCNU, $PGH_2 \rightarrow PGE_2$, H_2O_2
GSTM4-4	*GSTM4*	1p13.3	Cytosolic	Tyr6	CDNB
GSTM5-5	*GSTM5*	1p13.3	Cytosolic	Tyr6	CDNB
GSTP1-1	*GSTP1*	11q13	Cytosolic	Tyr7	acrolein, base propenals, BPDE, CDE, Chlorambucil, COMC-6, EA, Thiotepa,
GST1-1	*GSTT1*	22q11.2	Cytosolic	Ser11	BCNU, butadiene epoxide, CH_2Cl_2, EPNP, ethylene oxide
GST2-2	*GSTT2*	22q11.2	Cytosolic	Ser11	CuOOH, menaphthyl sulfate
GSTZ1-1	*GSTZ1*	14q24.3	Cytosolic	Ser13	dichloroacetate, fluoroacetate, 2-chloropropionate, malelyacetoacetate
GSTO1-1	*GSTO1*	10q24.3	Cytosolic	Cys32	monomethylarsonic acid, dehydroascorbic acid
GSTO2-2	*GSTO2*	10q24.3	Cytosolic	Cys32	dehydroascorbic acid
GSTS1-1	*GSTS1/ PGDS2*	4q22.3	Cytosolic	Tyr7	$PGH_2 \rightarrow PGD_2$
GSTK1-1	*GSTK1*	7q34	Mitochondria	Ser16	CDNB, CuOOH, (S)-15-hydroperoxy-5,8,11, 13-eicosatetraenoic acid

FLAP	*ALOX5AP*	13q12	MAPEG	Inactive	nonenzymatic binding of arachidonic acid
LTC_4S	*LTC4S*	5q35	MAPEG	Tyr93/ Arg104	$LTA_4 \rightarrow LTC_4$
MGST1	*MGST1/ PGES1*	9q34.3	MAPEG	Arg126	CDNB, CuOOH, hexachlorobuta-1,3-diene, $PGH_2 \rightarrow PGE_2$
MGST2	*MGST2*	4q28.3	MAPEG	Tyr93/ Arg104	CDNB, $LTA_4 \rightarrow LTC_4$, (S)-5-hydroperoxy-8,11, 14-*cis*-6-*trans*-eicosatetraenoic acid
MGST3	*MGST3*	1q23	MAPEG	Tyr93/ Arg104	CDNB, $LTA_4 \rightarrow LTC_4$, (S)-5-hydroperoxy-8,11, 14-cis-6-trans-eicosatetraenoic acid

Table 2. Description of human GSTs and their enzymatic properties.

Adapted from Hayes et al 2005, Mannervik et al 2005 and Higgins and Hayes 2011. Abbreviations: Δ5-ADD, Δ5-androstene-3,17-dione; AFB1, aflatoxin B1; BCDE, benzo[*g*]chrysene diol epoxide; BCNU, 1,3-*bis*(2-chloroethyl)-1-nitrosourea; BPDE, benzo[*a*]pyrene diol epoxide; BPhDE, benzo[*c*]phenanthrene diol epoxide; CDE, chrysene- 1,2-diol 3,4-epoxide; CDNB, 1-chloro-2,4-dinitrobenzene; CH_2Cl_2, dichloromethane; COMC-6, crotonyloxymethyl-2-cyclohexenone; CuOOH, cumene hydroperoxide; DBADE, dibenz[*a,h*]anthracene diol epoxide; DBPDE, dibenzo[*a,l*]pyrene diol epoxide; EA, ethacrynic acid; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; LTA_4, leukotriene-A_4; LTC_4, leukotriene-C_4; N-a-PhIP, N-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; NBD-Cl, 4-chloro-7-nitrobenzofurazan; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PGF_{2a}, prostaglandin F_{2a}; PGH_2, prostaglandin H_2;

1.4 GSTP1

1.4.1 *GSTP1* gene and regulation

Of the major cytosolic classes, GST Pi (π) is the most prominent extra-hepatic GST in humans and was originally discovered as a placental isoform (Dao et al., 1984) but was found to be largely expressed in most tissues in humans with the exception of the testes (Campbell et al., 1990), retina (Singh et al., 1985) and liver (Hiley et al., 1988). GSTP1 is highly conserved among species and is involved in a number of metabolic pathways in addition to its role in drug metabolism. In most species a single gene codes for GSTP1, *GSTP1*, which contains 7 exons and, in humans, is located on chromosome 11q13.2. There are a few organisms which are unusual in that they contain a second gene, *Gstp2,* upstream of the respective *Gstp1* gene such as mice and zebrafish, which is discussed further in Chapter 3. Like many other Phase II enzymes, GSTP1 is transcriptionally activated through Nrf2 binding to its ARE as discussed previously. Rats differ slightly as they contain a regulatory element known as a GSTP1 Enhancer 1 (GPE1) motif in the 5' upstream region (Sakai et al., 1988) which partly resembles an ARE sequence (Favreau and Pickett, 1995, Higgins and Hayes, 2011). From *in vivo* studies in rat liver, GPE1 is bound by transcription factors C/EBPα and CA-150 in quiescent liver cells, which suppress *GSTP1* transcription. During hepatocarcinogenesis, GPE1 is activated through Nrf2 binding (Sakai and Muramatsu, 2007) which can also be co-activated through MafK interaction with a histone acetyltransferase, the monocytic leukaemia zinc-finger protein (MOZ) (Ohta et al., 2007).

In addition to ARE activation by electrophiles, other regulatory elements have been identified in the human *GSTP1* gene and its promoter region and are highlighted in Figure 1.3. Moffat et al demonstrated the presence of an AP-1 site embedded in the ARE sequence which was

found to be activated by a Fos-Jun complex (Moffat et al., 1994) which supported previous observations made by Xia et al on the requirement of an AP-1 binding site for transcriptional activity of *GSTP1* (Xia et al., 1991). The binding to AP-1 was found to increase after stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA), hydrogen peroxide, doxorubicin and tert-butylhydroquinone (tBHQ) (Duvoix et al., 2004). Further work by Moffat and colleagues demonstrated the presence of an active Sp-1 site (Moffat et al., 1996) while Xia et al showed that GSTP1 transcription is suppressed upstream by retinoic acid but can be induced through an insulin binding site in intron 1 of *GSTP1* (Xia et al., 1993). Further work from the same group demonstrated that hydrogen peroxide induces *GSTP1* transcription through a putative NF-κB binding motif upstream of *GSTP1* (Xia et al., 1996). In contrast to the data provided by Xia and colleagues, Lo et al demonstrated that retinoic acid binding to a retinoic acid response element (RARE) in intron 5 of *GSTP1* results in increased transcriptional activity of a *GSTP1*C* haplotype (Lo and Ali-Osman, 1997). The same group subsequently identified a p53 binding motif in intron 4 of *GSTP1* which is thought to be transcriptionally active (Lo et al., 2008); the nature of this motif is discussed later in Sub-chapter 5.

As recently highlighted by Higgins and Hayes, the characterisation of *GST* genes at a molecular level is not as extensive within the literature as their regulation as a result of carcinogenesis and chemical stimulation (Higgins and Hayes, 2011). Although a number of regulatory motifs have been identified in *GSTP1*, some are yet to be fully characterised (see Chapter 5 (supplementary study)) while a number of regulatory motifs such as the Fork Head Protein (FKHD) and Paired box protein Pax-5, Erythroid Krüppel-like Factor (EKLF) binding sites have been predicted for human *GSTP1* using computational analysis (Vasieva, 2011). *GSTP1* is also subject to regulation through methylation of CpG islands upstream of

GSTP1 leading to reduced transcription of GSTP1. This is particularly apparent in prostate cancer where GSTP1 expression is absent (Lee et al., 1994). Treatment of prostate cancer cells with a DNA methyltransferase or histone deactetylase (HDAC) inhibitor restores GSTP1 expression (Hauptstock et al., 2011, Lin et al., 2001) while it has been demonstrated that the treatment of cells with maspin, a serine protease inhibitor, inhibits HDAC1 to restore GSTP1 expression (Li et al., 2011). Interestingly, mice in which the murine *Gstp* gene cluster is deleted and replaced with the orthologous human *GSTP1* gene demonstrate a CpG methylation pattern in mouse liver similar to that of human adult cells (Vaughn et al., 2011). However, this did not account for the lack of GSTP1 expression in the livers of these mice and therefore other *cis*-regulatory elements may explain differences in expression.

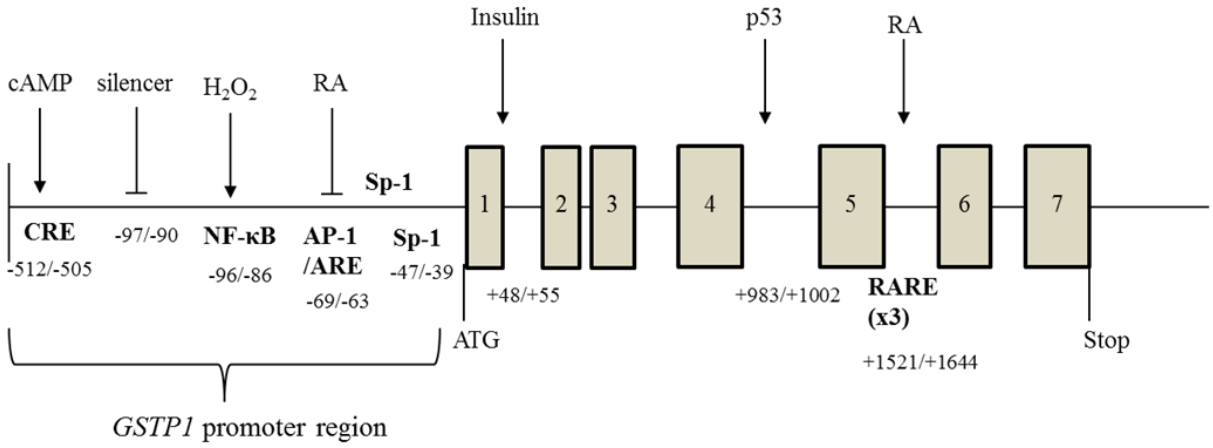


Figure 1.3. Schematic diagram of the human *GSTP1* gene and its promoter region with regulatory elements highlighted.

CRE, cAMP response element; ARE, antioxidant response element; RA, retinoic acid, RARE, retinoic acid response element. Adapted from Henderson et al 2011.

1.4.2 GSTP1 and carcinogenesis

GSTP1 has attracted much attention because of its apparent role in carcinogenesis and anticancer drug resistance. In 1989, Satoh et al. discovered that rats treated with dimethylnitrosamine developed hepatic neoplastic foci formation which were GSTP1 positive, a remarkable finding as rat liver contains very low levels of GSTP1 basally (Satoh et al., 1989). Work since then has shown that GSTP1 expression is elevated in most human tumours (Howie et al., 1990) and is believed to contribute to anticancer drug resistance as it is often found overexpressed in a wide range of cell lines made resistant to a diverse set of chemicals, some of which are not known to be GSTP1 substrates (Whelan et al., 1992, Wareing et al., 1993, Black et al., 1990). Many cancer drugs form electrophilic species after metabolism and are therefore subject to conjugation with GSH. Interestingly, transfection of GSTP1 cDNA into GSTP1 null cancer cell lines increased the resistance of a number of compounds which are not known substrates for GSTP1 (Moscow et al., 1989, Tew, 1994). Similarly, silencing or chemical inhibition of GSTP1 increases the sensitivity of a large number of cells to chemically induced apoptosis (Huang et al., 2007, Yu et al., 2009, McCaughan et al., 1994) and reduces proliferation of cancer cells (Dang et al., 2005, Hokaiwado et al., 2008). In contrast, mice nulled for *Gstp1* and *Gstp2* demonstrate increased tumour formation compared to their wild-type counterparts in response to a wide range of carcinogenic and genetic stressors which is discussed further in Chapter 3. As such, there is much interest in the role of this enzyme in contributing to cytoprotection and examining cellular functions of this enzyme other than drug metabolism.

1.4.3 GSTP1 and redox regulation

Redox signalling pathways are important in the regulation of cellular and thiol homeostasis, perturbations of which can lead to increased oxidative stress and cytotoxicity. Increased

production of reactive oxygen species (ROS) can lead to a number of disease states associated with increased oxidative stress such as Alzheimer's disease and has been attributed as a major cell signalling pathway in cancer. Cancer cells produce elevated levels of peroxides which have been found to contribute to their proliferation and activation of cell signalling cascades (Jones, 2008, Szatrowski and Nathan, 1991). Although not regarded as the primary enzymes involved in free radical scavenging, GSTs demonstrate activity towards a large number of oxidative species, either through direct conjugation of the species, or through modification of protein thiols and cell signalling cascades. Recent studies surrounding GSTP1-mediated cell regulation suggests that it plays a functional role, not only in preventing apoptosis through regulation of cell signalling cascades, but in maintaining cellular homeostasis through sulfhydryl protection in the prevention of oxidative stress (Townsend et al., 2006, Townsend et al., 2008a). Posttranslational modification of protein thiol groups, known as protein S-glutathionylation, is an important mechanism in preventing protein oxidation and in maintaining redox control. Protein S-glutathionylation is an interesting area of research in GSTP1 mediated cell regulation and this topic is discussed in detail in Chapter 4.

1.4.4 Non-catalytic functions of GSTP1

There is a growing body of evidence to suggest that GSTP1 can mediate cell regulation independently from its catalytic activity. A number of proteins and signalling molecules have been found to co-immunoprecipitate or interact with GSTP1 independently of its catalytic activity and are highlighted in Table 3. The majority of these interactions are thought to prevent against stress-apoptosis *in vitro*, although there is some *in vivo* evidence to suggest such regulatory pathways are functional basally (Elsby et al., 2003). Although there are a relatively small number of peer-reviewed papers describing some of these interactions, a

number of targets pertaining to GSTP1 regulation have been described for over a decade and are discussed in detail in later chapters of this thesis.

Interacting target	**Description**	**References**	**Chapters in which this function is described/discussed**
c-Jun N-terminal kinase (JNK)	GSTP1 binds to JNK as a monomer preventing phosphorylation of its downstream targets.	(Adler et al., 1999) (Wang et al., 2001b) (Elsby et al., 2003) (Thevenin et al., 2011)	Chapter 3 Chapter 4
Tumour necrosis factor receptor-associated factor 2 (TRAF2)	GSTP1 binds to TRAF2 and prevents ASK1 cell induced apoptosis.	(Wu et al., 2006)	ND
Peroxiredoxin (Prdx) VI	GSTP1 heterodimerises with PrdxVI resulting in PrdxVI S-glutathionylation and regeneration of its activity.	(Manevich et al., 2004) (Ralat et al., 2006)	Chapter 4
Nitric oxide (NO)	Acts as a NO carrier and forms a complex with ferrous irons to produce dinitrosyl-diglutathionyl iron complex (DNIC) in transporting cellular NO.	(Lo Bello et al., 2001) (Cesareo et al., 2005) (Lok et al., 2012)	Chapter 4
Protein thiols	Catalyses the S-glutathionylation of cellular proteins in response to nitrosative and oxidative stress.	(Townsend et al., 2008a) (Anathy et al., 2012)	Chapter 4
Cyclin dependent kinase-5 (Cdk5)	Inhibits Cdk5 activity through binding to Cdk5 and indirectly	(Sun et al., 2011)	ND

	through its peroxidase activity.		
Fanconi anemia group C protein (FANCC)	Interaction with FANCC inhibits apoptosis and prevents oxidation of GSTP1 Cys residues.	(Cumming et al., 2001)	ND
Transglutaminase 2 (TGM2)	Binding of GSTP1 with TGM2 inhibits GSTP1 activity.	(Piredda et al., 1999)	ND
Human Papillomavirus (HPV)-16 E7	HPV-16 E7 can dock to a GSTP1 monomer and enhances cell survival after UV exposure.	(Mileo et al., 2009)	ND
Death Receptor Fas	Interaction with GSTP1 results in Fas S-glutathionylation	(Anathy et al., 2012)	ND

Table 3. Non-catalytic properties of GSTP1.

The table highlights a number of cellular targets which are believed to interact with GSTP1, some of which are discussed in later chapters of the thesis. ND= not described in this thesis.

From Table 3 it is evident that the functions of GSTP1 extend well beyond enzymology and are related, either directly or indirectly, to the prevention of apoptosis and promotion of cell survival. Although much of the data surrounding GSTP1 cell regulation focuses on JNK signalling (see Chapters 3 and 4) Wu et al demonstrated that GSTP1 may possess other regulatory functions upstream of the MAP Kinase cascade by binding to tumour necrosis factor receptor-associated factor 2 (TRAF2) which prevents its association with apoptosis signal-regulating kinase 1 (ASK1) and reduces cell apoptosis (Wu et al., 2006). This followed earlier work showing that GSTP1 could coordinate ROS stimulated ASK1 signalling when transfected *in vitro* (Yin et al., 2000), suggesting regulation of these pathways may be

oxidative stress dependent. Mileo et al demonstrated that regulation of Map Kinase pathways by GSTP1 could be mediated through the binding of GSTP1 as a monomer to the Human Papillomavirus (HPV)-16 E7, which greatly reduced the number of apoptotic cells after UV treatment and enhanced HaCaT cell survival (Mileo et al., 2009). Interestingly, there is some evidence to suggest that GSTP1 may be phosphorylated by epidermal growth factor receptor (EGFR) and the Ser/Thr protein kinases, cAMP-dependent protein kinase A and C (PKA and PKC), although further studies are required to validate these findings (Lo et al., 2004, Okamura et al., 2009).

The identification of non-catalytic functions in other GST classes demonstrates that this is an intrinsic function to GST in cell regulation and not exclusive to GSTP1. A number of GST isoenzymes are able to bind 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 (15-d-PGJ_2) which prevents its translocation to the nucleus and activation of peroxisomal proliferator-activated receptor γ (PPARγ) (Paumi et al., 2004). The GSTA isoenzyme has been implicated in the transport of bilirubin (Kamisaka et al., 1975, Simons and Jagt, 1980) and steroid synthesis (Johansson and Mannervik, 2001) although it is uncertain whether or not these reactions can occur non-catalytically. As described in Table 3, there is much evidence to suggest GSTP1 can inhibit JNK activity in mediating cellular apoptosis (discussed in later chapters). Further evidence to support GST regulation of kinase activity is demonstrated through the inhibitory effect of GSTM1 on the stress activated MAP Kinase cascade. GSTM1 is the most closely related class of GST to Pi and can function as an inhibitory regulator of the MAP Kinase Kinase Kinase (MAPKKK) pathway by binding to ASK1 and MAP/ERK Kinase Kinase 1 (MEKK1) and protect against ASK1 dependent apoptosis (Cho et al., 2001, Ryoo et al., 2004). The extent to which this occurs *in vivo* requires careful examination as around 50% of the Caucasian population are null for *GSTM1* and therefore may lack this form of regulation

(Hirvonen et al., 1993). Yin et al also demonstrated that GSTs were found to bind to the serine protease inhibitor (serpin), Maspin, and that GSTM3 was strongly associated with its interaction as determined through a Yeast two hybrid screen (Yin et al., 2005). The key finding from these studies is that signalling regulation was found to be independent of their glutathione-conjugating abilities, suggesting that the non-catalytic binding of GSTs may mediate an important function in protecting cellular proteins and in the regulation of stress response signalling pathways. The ability of GSTP1 to function non-catalytically raises fundamental questions as to how this enzyme contributes to cell regulation and what role it plays in tumorigenesis and cancer progression.

1.5 Aims of study

It is clear that there are a number of functions relating to GSTP1 that are important in mediating anticancer drug resistance and cancer proliferation which are not dependent on its catalytic activity. As yet, there are no *in vivo* models to fully assess the nature of these properties, while some of the relationships between GSTP1 and cellular proteins have only been postulated in a limited number of cellular models or from individual laboratories. This study aims to assess the non-catalytic functions of GSTP1 and examine the extent to which they may contribute to cell regulation. The first part of this study describes the characterisation of a novel *in vivo* model in which the catalytic activity of GSTP1 has been removed, and assesses the role of this enzyme in response to a well-characterised hepatotoxin, acetaminophen. The second part of this thesis will investigate some of the non-catalytic functions of GSTP1 *in vitro*, particularly in relation to sulfhydryl homeostasis and redox regulation. Finally, this study will aim to examine novel pathways and protein targets which may be mediated by GSTP1.

2. Materials and Methods

2.1 Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma or VWR. Cell culture media was purchased from Invitrogen.

2.2 Animals

2.2.1 Husbandry

All animal studies were carried out in accordance with the Animal (Scientific Procedures) Act 1986 and approved by the Animal Ethics Committee of the University of Dundee. Mice were housed in open top cages and exposed to a 12 hour light/dark cycle, with *ad libitum* access to water and, unless stated otherwise, RM1 standard animal diet.

Gstp1 wild-type (*Gstp1*^{WT}) and *Gstp1/2* null (*Gstp1/2*$^{-/-}$) mice were generated as previously reported (Henderson et al., 1998b) and backcrossed onto a C57/BL6J background for at least 8 generations.

2.2.2 Sacrifice

Unless stated otherwise, for all *in vivo* experiments mice were sacrificed by exposure to a rising concentration of carbon dioxide. Where appropriate, blood was taken from cardiac punctures into heparinised tubes and centrifuged. The plasma was removed, snap frozen in

liquid nitrogen and stored at -80°C. Details of protocols for handling other animal tissues are described elsewhere.

2.3 Animal Drug Treatments

2.3.1 Paracetamol (acetaminophen)

Paracetamol (APAP) tablets were dissolved by sonication in phosphate buffered saline (PBS) at 15mg/ml. Mice were starved for 16 hours prior to APAP administration by oral gavage at 300mg/kg body weight.

2.3.2 Buthionine sulfoximine

Buthionine sulfoximine **(**BSO) was dissolved in sterile water at 30mg/ml. Mice were administered a single intraperitoneal (i.p) dose of BSO at either 0.9g/kg or 0.2g/kg body weight.

2.4 Generation of GSTP1 Y7F mutant mouse model

Mice harbouring a tyrosine to phenylalanine mutation at codon 7 in the *Gstp1* gene were generated by TaconicArtemis, Cologne, Germany. The targeting vector was assembled using mouse genomic fragments obtained from the C57Bl/6J RPCIB-731 BAC library which introduced a Y7F mutation into exon 2 of the *Gstp1* gene, and loxP sites flanking exon 1 to 4 of the *Gstp2* gene, including 1kb of the promoter region. The targeting vector was linearized with *Sfi* I and transfected into C57BL/6NTac mouse embryonic stem (ES) cells, where positive clones were identified under puromycin selection. Validation of homologous recombinant ES cell clones was performed using Southern blotting and PCR screening. ES

cells were injected into the blastocysts of superovulated BALB/c female mice before being implanted into pseudopregnant NMRI females. Resultant offspring were assessed for chimaerism and highly chimaeric offspring (G0) were subsequently crossed with C57Bl/6J females. Germline transmission was identified by the presence of black (C57Bl/6J strain) offspring. Mice heterozygous for the targeted locus (*Gstp1*$^{WT/Y7F}$) were subsequently crossed to produce homozygous breeding lines (*Gstp1*$^{Y7F/Y7F}$).

2.5 Isolation of primary mouse embryonic fibroblasts (MEFs)

Pregnant female mice were euthanized by cervical dislocation between days 12-14 of pregnancy. Embryos were aseptically removed and washed in ice cold PBS. The brain and internal organs were removed and the remaining embryo was finely scissor minced and incubated at 37°C for 5 minutes in 9ml of Trypsin-EDTA (Invitrogen). 9 ml of MEF medium (DMEM, 10% FBS, 2mM L-glutamine and 100U/ml penicillin, 100µg/ml streptomycin) was then added to neutralise the Trypsin-EDTA. The mixture was transferred to a 50 ml Falcon tube and centrifuged at 800xg for 5 minutes. The supernatant was removed and the pellet was resuspended in 5 ml of Trypsin-EDTA and left at 37°C for 10 minutes. MEF medium (20ml) was added to disaggregate the tissue and the debris was allowed to settle before plating the supernatant onto sterile 15 cm culture dishes. Cells were incubated at 37°C/5% CO_2 overnight. The following morning cells were washed with sterile PBS, replaced with fresh MEF medium and incubated overnight. Cells were trypsinised and resuspended into 30 ml of MEF medium. 10 ml of suspension was aliquoted into 3x 10cm culture dishes. Over the next few days cells were allowed to grow to 95-100% confluency before being seeded for experiments or frozen down in 10% DMSO in fetal bovine serum (FBS).

2.6 Molecular cloning

2.6.1 Cloning of Mouse *Gstp1*^{WT} and *Gstp1*^{Y7F} constructs

Full length mouse *Gstp1* cDNA was kindly provided by Aileen McLaren, University of Dundee. Due to the proximity of the Y7F mutation to the starting codon, site directed mutagenesis was performed using a single round of PCR. The *Gstp1*^{Y7F} mutation was introduced into the *Gstp1* reading frame, while *Nde1* and *BamH1* restriction sites were introduced at the 5' and 3' termini respectively; *Gstp1*^{WT} primer (CGC ATA TGC CAC CAT ACA CCA TTG TCT ACT TCC CAG TTC GAG GGC G), *Gstp1*^{Y7F} primer (CGC ATA TGC CAC CAT ACA CCA TTG TCT TCT TCC CAG TTC GAG GGC G), reverse primer (GCG GAT CCC TAC TGT TTG CCA TTG CCA TT). All PCR reactions were performed using 5µl *Pfu* polymerase reaction buffer (20mM Tris pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100 and 0.1mg/ml nuclease-free BSA), 0.2mM dNTP mix, 5ng cDNA, 15 pmol of each primer and 1.5 units of *Pfu* polymerase. The reaction was carried out using a Biometra T3000 Thermocycler as follows; one cycle at 95°C for 30 seconds followed by 35 cycles of 95°C for 30 seconds, 60°C for 1 minute and 68°C for 1 minute. The reaction was run on a 1% agarose gel at 100mV for 45 minutes and the PCR product was excised and extracted. The PCR product was ligated into pCR™-blunt before being transformed into One Shot® Top10 Chemically Competent *E. Coli* cells. Transformations were spread onto agar plates containing kanamycin (50µg/ml) and incubated overnight. Plasmid DNA was extracted from colonies formed and sequenced to confirm the successful addition of restriction sites and introduction of mutation.

From successful colonies, mouse *Gstp1*^{WT} and *Gstp1*^{Y7F} inserts were cloned into the *Nde1* and *BamH1* sites of the pET11a vector downstream of the *lac* operon. Inserts were digested at

37°C for 2.5 hours in a buffer containing 1x Promega Buffer D (6mM Tris-HCl pH 7.9, 6mM $MgCl_2$, 150mM NaCl, 1mM DTT), 0.1mg/ml BSA, 15 units of *Nde1* and 30µl DNA. The digest was then heated at 65°C for 10 minutes to inactivate the enzyme. The reaction was cleaned and purified using the PureLink® PCR Purification Kit (Invitrogen) according to the manufacturer's protocol, and inserts were eluted in 40µl purified water. Inserts were subsequently digested with *BamH1* using the same protocol, except using 1x Buffer E (6mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 100mM NaCl, 1mM DTT) in place of Buffer D. The incubation was run on a 1% agarose gel and the digested insert was excised and extracted. The insert was ligated into pET11a using T4 ligase. 3µl of plasmid was transformed into BL21-Gold(DE3)pLysS competent *E. Coli* cells (Stratagene). Colonies formed from the transformation were sequenced to determine correct cloning of inserts, and successful constructs were used to produce recombinant protein.

2.6.2 Recombinant protein induction and purification

Bacterial cells containing mouse $Gstp1^{WT}$ and $Gstp1^{Y7F}$ constructs were grown overnight in 20ml of LB medium at 37°C, 180rpm. An aliquot (5ml) of this culture was added to 500ml of LB medium and incubated for 2.5 hours at 37°C, 180rpm. Recombinant protein expression was induced with 1mM IPTG for 2 hours before cultures were centrifuged at 4,500rpm for 20 minutes at 4°C. A fraction of culture was removed prior and after induction and analysed on a SDS-PAGE gel to confirm protein induction. Pellets were resuspended in 15ml of Buffer A (50mM Tris-HCl, pH 7.4, 200mM NaCl, 0.5mM DTT). The suspension was mixed with lysozyme (100µg/ml) and incubated for 15 minutes at 30°C. Protease inhibitors were added and the lysate was rotated for 30 minutes at 4°C. Lysates was sonicated for 3x 1minute intervals (14 amp, pulse 10 seconds) with 1 minute on ice between sonications. Lysates were

centrifuged at 40,000rpm for 30 minutes at 4°C and the resultant suspension filtered (0.45µm).

Purification of recombinant mouse GSTP1 was performed using GSTrap™ FF columns (GE Healthcare). Columns were equilibrated with 5 column volumes of water at 1ml/min and then 5 column volumes of Buffer A at 1ml/min. 100µl lysate was removed from the preparation and the remaining lysate was passed over the column at 0.25ml/min and the flow through collected. The column was washed with 15 column volumes of Buffer A at 1ml/min. Bound protein was eluted from the column using Elution buffer (200mM Tris-HCl pH 7.9, 50mM glutathione) and collected in 10x0.5ml fractions. To determine the presence of recombinant protein in each fraction, 5µl of each fraction, as well as flow through and lysate taken prior to purification were subjected to SDS-PAGE analysis. Recombinant protein was identified through Coomassie Blue staining and fractions containing recombinant GSTP1 were isolated and combined before being desalted. Desalting columns (BioRad) were equilibrated with 2 column washes of 50mM Tris pH 7.4. 3ml of lysate containing recombinant GSTP1 was added to the column and allowed to flow through. The protein was eluted using 4ml 50mM Tris pH 7.4 and stored at -80°C.

2.6.3 GFP-tagging of human GSTP1^{WT}, GSTP1^{Y7F} and GSTP1 ^{V105I} constructs

The open reading frame of human *GSTP1*, derived from HCT116 cDNA, had previously been cloned into pcDNA3.1 and was used as a template for cloning into the peGFP-C3 vector. Genes cloned into this vector are expressed as fusions to the C-terminus of GFP. The cloning strategy was divided into two parts. Initially, silent mutations were introduced into the *GSTP1* open reading frame to prevent silencing of the gene by shRNA, along with the

introduction of *EcoR1* and *BamH1* restriction sites at the 5' and 3' ends, respectively. The silent mutations introduce base changes in the shRNA binding region but do not result in a change to the amino acid sequence, as shown below:

GSTP1 reading frame	GGA GAC CTC ACC CTG TAC CAG TCC AAT ACC
Silent mutation	GGA GAC CTC ACT TTA TAT CAG TCC AAT ACC
Amino acid	G D L T L Y Q S N T

The cloning was performed using a two-step PCR procedure as described previously (Landt et al., 1990). All PCR reactions were carried under the buffer and cycling conditions described for the cloning of mouse $Gstp1^{WT}$ and $Gstp1^{Y7F}$ constructs. The first PCR reaction introduced the silent mutations into the *GSTP1* open reading frame; silent mutation primer (GGA GAC CTC ACT TTA TAT CAG TCC AAT ACC), reverse primer (GAG TCC CCC GGA TCC TCA CTG TTT CCC GTT GCC). The base changes resulting in silent mutations are underlined. The product from this reaction was run on a 1% agarose gel, excised and extracted, and then used as the reverse primer in a second PCR reaction, to generate the wild-type *GSTP1* open reading frame containing silent mutations and restriction sites; forward primer (GCC GCC GCA GTC AGA ATT CCC ATG CCG CCC TAC ACC GTG). The reaction was ligated into pCR™-blunt before transformed into One Shot® Top10 Chemically Competent *E. Coli* cells. Colonies were sequenced, with correct constructs subsequently cloned into peGFP-C3. Inserts were digested at 37°C for 3 hours in a buffer containing 1x Promega Buffer E (6mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 100mM NaCl, 1mM DTT), 0.1mg/ml BSA, 20 units of *EcoR1,* 20 units of *BamH1* and 40µl DNA. The digest was then

heated at 65°C for 10 minutes to inactivate the enzymes. The reaction was run on a 1% agarose gel and the insert was excised and extracted, before ligated into peGFP-C3. 3µl plasmid was transformed into NEB 10-beta Competent *E. Coli* cells (New England BioLabs) and colonies sequenced for successful introduction of the *GSTP1* open reading frame into peGFP-C3 (*GSTP1*^{WT}-GFP).

The second part of the cloning strategy involved introducing Y7F and V105I mutations into the *GSTP1* open reading frame. This was achieved using the Stratagene QuickChange™ Kit and the following primers; Y7F (forward, CCG CCC TAC ACC GTG GTC TTC TTC CCA GTT CGA GGC CG, reverse CGG CCT CGA ACT GGG AAG AAG ACC ACG GTG TAG GGC GG), V105I (forward, GCT GCA AAT ACA TCT CCC TCA TCT ACA CCA ACT ATG AGG, reverse CCT CAT AGT TGG TGT AGA TGA GGG AGA TGT ATT TGC AGC). The PCR reactions were carried out using the *GSTP1*^{WT}-GFP plasmid as a template under the following conditions; 1x QC Reaction Buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl pH 8.8, 2mM $MgSO_4$, 0.1% Triton X-100, 0.1mg/ml nuclease free BSA), 125ng each primer, 50ng cDNA, 0.2mM dNTP mix and 2.5 units of *PfuTurbo* DNA polymerase. The reaction was carried out using a Biometra T3000 Thermocycler as follows; one cycle at 95°C for 30 seconds followed by 17 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 6 minutes. 1µl (10 units) of *Dpn* I was added to the reaction and left to incubate for 1 hour at 37°C. This endonuclease is specific for methylated and hemimethylated DNA, digesting the parental DNA template but not the mutated vector. 3µl of the reaction was transformed into NEB 10-beta Competent *E. Coli* cells and colonies sequenced for the successful introduction of Y7F and V105I mutations into peGFP-C3 (*GSTP1*^{Y7F}-GFP and *GSTP1*^{V105I}-GFP respectively).

2.6.4 Agarose gel extraction of PCR products

The extraction of PCR products from agarose gels was performed using the PureLink® Quick Gel Extraction Kit according to the manufacturer's instructions.

2.6.5 DNA sequencing

DNA sequencing of colonies were analysed using Capillary Electrophoresis by the Genetics Core Services, University of Dundee.

2.6.6 Extraction of DNA from bacterial colonies

Isolation of plasmid DNA from bacterial cultures was performed using the PureLink™ Quick plasmid Miniprep or HiPure Plasmid Maxiprep kit. For minipreps, bacterial cells were grown overnight in 10ml of LB medium containing antibiotic selection. The following morning, cultures were pelleted at 3000xg for 10 minutes at 4°C, and plasmid DNA was isolated according to the manufacturer's protocol. For maxipreps, 5ml of culture was grown in antibiotic selection for 8 hours before 1ml was transferred to 200ml LB medium containing antibiotic selection and grown overnight. The cultures were pelleted at 3000xg for 10 minutes at 4°C and plasmid DNA was isolated according to the manufacturer's protocol. Plasmid DNA was stored at -20°C.

2.6.7 Ligation of PCR products into plasmid vectors

For the ligation of PCR products into the pCR™-blunt Vector, 3µl of PCR product was incubated with 25ng pCR™-Blunt vector, 4 units of T4 DNA ligase and 1x T4 DNA Ligation

buffer (6mM Tris-HCL, pH8, 6mM $MgCl_2$, 5mM NaCl, 0.1mg/ml BSA, 7mM ß-mercaptoethanol, 0.1mM ATP, 2mM DTT, 1mM spermidine) for 1 hour at 16°C.

For the ligation of PCR products into pET11a and peGFP-C3 vectors, 7µl of PCR product was incubated with 3 units of T4 DNA ligase and 1µl of T4 DNA ligase buffer (30mM Tris-HCL, pH8, 10mM $MgCl_2$, 1mM ATP, 10mM DTT) for 16-18 hours at 16°C.

2.6.8 Transformation of plasmids into bacterial cells

Ligation product (3µl) was incubated with 50µl of One Shot® Top10, BL21-Gold(DE3)pLysS or NEB 10-beta Chemically Competent *E. Coli* cells for 30 minutes on ice. Cells were heat shocked for 45 seconds at 42°C before incubated on ice for 2 minutes. 450µl of LB medium was added to the cells which were incubated with agitation at 37°C for 1 hour. Transformations were spread onto bacterial culture plates with an antibiotic selection and incubated overnight.

2.7 Kinetic assays

The enzymatic activities of recombinant GSTP1 was determined spectrophotometrically at 25°C, using 1-chloro-2,4-dinitrobenzene (CDNB) as a co-substrate as described by Habig (Habig et al., 1974). Kinetic assays were performed in 0.1M potassium phosphate buffer, pH 6.5. Initially, the linear rate of reaction was determined using variable concentrations of protein (0.78-50µg) using fixed concentrations of GSH (10mM) and CDNB (2mM). The apparent Km^{CDNB} was determined using a fixed concentration of 10mM GSH and variable concentrations of CDNB (0.01–2mM CDNB). The apparent Km^{GSH} was determined using a

fixed concentration of CDNB (1mM) and variable concentrations of GSH (0.08-10mM). V_{max} and K_m values were derived fromMichaelis-Menton kinetic curves generated using GraFit 5 (Erithacus Software).

2.8 Cell culture

2.8.1 Cells

HCT116 cells were kindly provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD). HCT116 cells were maintained in McCoy's 5A media supplemented with 10% FBS and 2mM L-glutamine. SAOS2 and SAOS2/p53 cells were kindly provided by Dr. David Meek (University of Dundee). SAOS2 and SAOS2/p53 cells were maintained in DMEM supplemented with 10% tet-free FBS and 2mM L-glutamine.

2.8.2 Transfection of HCT116 cells using siRNA

Transfection of siRNA into HCT116 cells was performed using Lipofectamine RNAiMAX. Cells were seeded onto 6 well, 6cm or 10cm tissue culture dishes, so that the density of the cells was 30% confluency 24 hours later. Depending on the size of plate to be transfected, 4-25µl RNAiMAX was incubated with Silencer® select (Ambion) siRNA targeting GSTP1 (#s306) or a negative control siRNA (#4390843) in Optimem, so that the final concentration of siRNA on the cells was 10nM. The reaction was incubated for 20 minutes at room temperature before added dropwise to cells. Cells were incubated overnight and media was changed the following morning.

2.8.3 Generation of GSTP1 stable knock down HCT116 cell line using lentiviral mediated shRNA

Stable knock down of GSTP1 in HCT116 cells was achieved using lentiviral transduction of short hairpin RNA constructs (shRNA) followed by clonal selection. For production of lentiviral particles, HEK293-T cells were seeded at $3x10^6$ cells per 10cm plate for 70-80% confluency 24 hours later. Four separate MISSION shRNA clones (Sigma) targeted against different regions of *GSTP1* mRNA transcript (2.6µg) were packaged with 26µg of Viral Packaging Mix (Sigma) and added to 182µl of serum free medium containing 16µl FuGENE transfection reagent. The hairpin sequences of each shRNA construct contain a 21 base stem and a 6 base loop which were cloned into the pLKO.1 vector and driven from the pol III U6 promoter. The Sigma Viral Packaging Mix contains the vesicular stomatitis virus G-protein (VSV-G) envelope vector for pseudotyping of lentiviral particles, and a packaging vector which encode the virion structural proteins. The transfection cocktail was incubated at room temperature for 15 minutes before added dropwise to culture dishes. Medium was replaced 16 hours post-transfection. The viral supernatant was harvested from cells at 36 and 72 hours post-transfection, filtered through 0.45µm filter and stored at -80°C.

For infection of HCT116 cells with lentiviral particles, cells were grown on 10cm culture dishes to 20-30% confluency. Polybrene was added to the dishes at a final concentration of 8µg/ml and increasing concentrations of viral supernatant (10-200µl) was added to cells. Cells were incubated for 72 hours before puromycin (3µg/ml) was added for clonal selection. Individual clones were picked and knockdown of *GSTP1* mRNA was determined by rtPCR. Cells were maintained in puromycin selection during culture. Puromycin was removed prior to the start of any *in vitro* experiments.

2.8.4 Transfection of plasmid DNA into cells

Transfection of plasmid DNA into HCT116 cells was performed using Lipofectamine™ LTX and PLUS™ reagents (Invitrogen). Depending on the size of culture dish to be transfected 1.25-4µg plasmid DNA was incubated with a 1:1 ratio of PLUS™ reagent in Optimem for 5 minutes at room temperature e.g. 2µl of PLUS™ reagent would be incubated with 2µg DNA. Lipofectamine™ LTX reagent was added at a 2.5:1 ratio with DNA and incubated for 30 minutes at room temperature. The transfection cocktail was added dropwise to cells which were grown to 70% confluency. Cell media was replaced after 6 hours, and the cells were incubated for a further 18 hours.

2.8.5 Cytotoxicity assays

HCT116 cells untreated, stably-silenced for GSTP1 or containing a control plasmid were seeded into a 96 well plate at 1500 cells per well in 100µl media. 24 hours after seeding, 100µl media containing a cytotoxic agent was added to each well. All drugs were dissolved in DMSO and diluted such that the final concentration of DMSO in the cell media was never more than 1%. Cells were incubated with drug for 72 hours before their cell number was determined using the ATP assay.

2.8.6 Determination of cell number using the ATP assay

The numbers of cells in a 96 well plate were determined using the ATP assay. The ATP assay is based on Firefly Luciferase catalysis of D-Luciferin, whereby the amount of light emitted from the reaction is proportional to the amount of ATP present as a result of its consumption

by Luciferin. Media was removed from cells and then incubated for 1 minute in 45µl of Somatic cell ATP releasing buffer (Sigma). 30µl of lysate was transferred to a white 96 well-assay plate. 30µl of ATP Assay Mix, diluted 1:25 in ATP Dilution Buffer (Sigma) was added to each well and amount of light emitted was detected on an Orion II Microplate Luminometer (Berthold Detection Systems).

2.8.7 UV treatment of cells

HCT116 cells were seeded into 6cm culture dishes and irradiated with defined doses of UV (254nm) using a Stratlinker (Stratagene) 72 hours post transfection of siRNA targeting GSTP1 or a control siRNA (10nM). Prior to irradiation cell media was removed and incubated at 37°C. Cells were overlaid with warm PBS (1ml) and irradiated. Cells were subsequently incubated with the same media removed prior to irradiation for a defined period of time.

2.9 Histology

Animal tissues were excised, washed in PBS and fixed in 4-10% formal saline histological fixative (Gurr). Tissues were left rocking gently in fixative for 24 hours at room temperature before embedded in paraffin wax. Tissues were cooled on ice for at least 1 hour prior to sectioning. For immunohistochemical and histological staining, sections were cut to 5µm and transferred to Polysine® slides.

2.9.1 Hematoxylin and eosin (H/E) staining

Tissue sections were deparaffinised in 2x 20 minute washes of xylene and rehydrated through 2 minute washes in decreasing concentrations of ethanol; 100% (x2), 95%, 90%, 70%, 50% ethanol. Sections were incubated for 2 minutes in PBS and then for 2 minutes in Gills Hematoxylin, before briefly reduced in acid alcohol. Sections were rinsed for 15 minutes in tap water and incubated for 2 minutes in 80% ethanol before stained briefly in eosin Y solution. Sections were dehydrated in increasing concentrations of ethanol, incubated in xylene solution and then left to dry. Slides were sealed using DPX mounting medium and left overnight.

2.9.2 Immunohistochemistry

Tissue sections were deparaffinised and rehydrated as described under 'H/E staining'. Antigen retrieval was achieved by boiling sections in sodium citrate buffer (0.01M, pH 6.5) for 20 minutes and then leaving to cool to room temperature. Endogenous enzymes were blocked for 10 minutes in Dual Endogenous Enzyme Block solution (DAKO). Sections were blocked in 10% goat serum in 0.1% Tween/PBS (PBST) for 30 minutes, washed in PBST and incubated with a primary antibody for 1 hour at room temperature. Secondary antibody and substrate-chromogen labelling was achieved using the DAKO EnVision and Dual-Link System-HRP kit according to the manufacturers protocol. Sections were washed in PBST and counterstained with Haematoxylin solution before dehydrated in increasing concentrations of ethanol. Sections were incubated in xylene solution before air dried and sealed using DPX mounting medium.

2.10 Microscopy

Fixed and frozen tissue sections were examined, and photomicrographs taken, using an AxioCam microscope with Axiovision software (Zeiss). Laser microscopy was performed using a LSM 510 confocal microscope (Zeiss).

Immunogold labelling of tissue and electron microscopy (EM) was carried out by the Microscopy Facility, College of Life Sciences, University of Dundee.

2.10.1 Immunogold labelling of mouse GSTP1

Livers from mice treated with APAP were harvested and cut into small pieces in 2% PFA solution. Livers were transferred to 30% sucrose solution, frozen and sectioned. The sections were collected on pioloform/carbon coated copper grids. For immunogold labelling, sections were floated on droplets of solutions in the following protocol.

Sections were washed twice in PBS and blocked with 0.5% Fish Skin Gelatin (FSG) in PBS for 10 minutes. Sections were incubated with neat GSTP1 primary antibody for 30 minutes before washed 3 times in PBS. Sections were then incubated for 20 minutes with Goat anti-rabbit gold secondary antibody (1:40). After 6x5 minute washes in PBS and one in distilled water, sections were incubated in 2%methyl cellulose/3% uranyl acetate for 2x1minute washes and a final 5 minute wash before dried and analysed by EM.

2.10.2 Transmission electron microscopy

$1x10^6$ HCT116 cells were seeded onto 10cm plates and transfected with 10nM of siRNA targeted for GSTP1, a non-targeting control siRNA or untransfected as described previously. 72 hours post transfection, cells were fixed using 2% PFA in 0.1M Cacodylate buffer, pH 7.4. Cells were post fixed in 1% aqueous osmium and dehydrated in graded alcohols before incubated in propylene oxide to remove residual ethanol. Cells were placed in neat resin and polymerised at 60°C before analysed by EM.

2.11 Flow Cytometry

All flow cytometry was performed using a Becton Dickson FACScan (Flow Cytometry Core Facility, University of Dundee).

2.11.1 Propidium Iodide (PI) staining

Cell cycle profiles of HCT116 cells were determined using Propidium Iodide (PI) staining. Cells were grown to 70% confluency, trypsinised and pelleted. Cell pellets were washed in PBS, pelleted and suspended in residual PBS solution. Cells were fixed in ice cold 70% ethanol and incubated at -20°C for at least 30 minutes. After fixation, cells were washed twice in PBS containing 1% bovine serum albumin (BSA). Cells were pelleted, resuspended in PI solution (50µg/ml propidium iodide, 50µg/ml RNase) prepared in PBS and incubated for 20 minutes at room temperature before analysed by flow cytometry.

2.11.2 Dichlorodihydrofluorescein Diacetate (H_2DCFDA) staining

H_2DCFDA is a reduced fluorescein which is readily converted to a green-fluorescent form in the presence of oxidants and therefore can be used as a measure of ROS activity in the cell. Cells were grown to 70% confluency and incubated with 10µM H_2DCFDA in PBS for 30 minutes at 37°C. Cells were trypsinised, and washed twice in PBS containing 1% BSA and finally resuspended in PBS before analysed by flow cytometry.

2.11.3 Apoptotic assays

Analysis of apoptotic pathways in HCT116 cells was performed using the Intellicyt Multimetric Apoptosis Screening kit. Cells were grown to 70% confluency and stained for a number of apoptotic pathways according to the manufacturer's protocol. Cells were incubated in 200µl Caspase 3/7 Detection Reagent, Mitochondrial Integrity Reagent, and Annexin V/Cell Viability Reagent. Cells were washed with PBS in between each staining step. For a positive control of apoptosis induction, HCT116 cells had been treated with 10µM staurosporine for 18 hours. Cells were analysed by flow cytometry using a Becton Dickinson LSRFortessa. The excitation wavelengths/peak emissions for the reagents are as follows; Caspase 3/7, 488/518; Mitochondrial Integrity, 638/658; Annexin V, 488/575; Cell Viability, 488/647.

2.12 RNA analysis

2.12.1 Extraction of RNA from mouse tissue

RNA was extracted from mouse tissue using TRIzol® and cleaned using RNeasy Minikit (Qiagen). Tissue (100mg) was homogenised at room temperature in 1ml of TRIzol®. Samples

were incubated for 5 minutes at room temperature before centrifuged at 10,000xg at 4°C. Chloroform (0.2ml) was then added to the supernatant and mixed thoroughly before incubated for 3 minutes at room temperature. Samples were centrifuged at 10,000xg for 15 minutes at 4°C and the supernatant added to 0.5ml isopropanol. Samples were incubated for 10 minutes at room temperature and then centrifuged at 10,000xg for 10 minutes at 4°C. The pellet was washed with 1ml of 75% ethanol and vortexed. Samples were pelleted again before left to air dry. The pellet was dissolved in 100µl RNAse-free water and incubated at 55°C for 10-15 minutes. The isolated RNA was then cleaned using the RNeasy Minikit according to the manufacturer's instructions. RNA was eluted in 50µl water stored at -70°C.

2.12.2 Extraction of RNA from cell cultures

RNA was extracted from cell cultures using the PureLink RNA Minikit (Ambion) according to the manufacturer's instructions. RNA was eluted in 50µl water stored at -70°C.

2.12.3 Reverse Transcription (RT) of isolated RNA for synthesis of cDNA

Reverse Transcription of isolated RNA was performed using the ImPromII Reverse Transcription Kit (Promega). Concentrations of RNA were determined spectrophotometrically using the NanoDrop ND-8000 Spectrophotometer. Purity of RNA was determined by calculating the ratio of absorbance at 260nm and 280nm (260/280 ratio) where values of ~2.0 were accepted as pure for RNA. A secondary measure at 260nm and 230nm (260/230 ratio) was also used to determined nucleotide purity, where values between 2.0-2.2 were accepted as pure for RNA. RNA was diluted to 200ng/µl and DNase treated with RQ1 DNase. 5µl of RNA was added to 1µl RQ1 buffer (40mM Tris-HCl pH 8.0, 10mM $MgSO_4$ and 10mM $CaCl_2$), 1µl RQ1 DNase and 3µl DEPC treated water. The samples were

incubated at 37°C for 30 minutes before the reaction was stopped by adding 1µl RQ1 stop buffer (2mM EGTA pH 8.0) and incubated at 65°C for 10 minutes. Random primers (1µl) were added to 4µl of DNase-treated RNA and incubated at 70°C for 5 minutes and then incubated on ice. For reverse transcription of RNA, a master mix composed of the following reagents was added to each sample; 4µl ImPromII reaction buffer, 1.2µl $MgCl_2$, 1µl dNTP mix, 0.5µl Ribonuclease Inhibitor, 1µl ImPromII Reverse Transcriptase and 7.3µl DEPC treated water. The samples were then incubated at 25°C for 5 minutes, 42°C for 1 hour and finally 70°C for 15 minutes. cDNA samples were stored at -20°C.

2.12.4 Real Time PCR (Taqman) analysis

Quantitation of gene expression was performed using the 7500 Real Time PCR System (Applied Biosystems). Reverse transcribed cDNA was diluted 1:80 in nuclease-free water and 8µl of diluted cDNA was added to a duplex reaction containing 1µl Taqman primer, 12µl Taqman Gene Expression Master Mix (Applied Biosystems) and 1µl 18S primer, which serves as an endogenous control. For duplex reactions, 18S was VIC-labelled while all other Taqman probes were FAM-labelled. The reaction was mixed and added in triplicate to a MicroAmp® Optical 96 well plate. The cycling parameters are as follows; 50°C for 2 minutes, 95°C for 10 minutes and an amplification step of 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute.

Results from Taqman analysis are displayed as fold difference from the control in each experiment. The difference in cycle threshold (Ct) values between primer probe and 18S are determined (ΔCt) and normalised against the 'calibrator' or control sample in each experiment (ΔΔCt). The fold difference is determined as 2 to the power of ΔΔCt ($2^{-\Delta\Delta Ct}$).

2.12.5 RNA amplification for microarray profiling

Generation of biotinylated amplified RNA (cRNA) for hybridization with Illumina® arrays was performed using the Illumina® TotalPrep™ RNA amplification kit with a T7 Oligo(dT) Primer to synthesise cDNA containing a T7 promoter sequence. DNase treated RNA (500ng) from HCT116 untreated, HCT116 GSTP1 shRNA 1, HCT116 GSTP1 shRNA 2 and HCT116 control plasmid was amplified according to the manufacturer's protocol. cRNA was eluted in 200µl of nuclease-free water and stored at -20°C.

2.12.6 Gene expression profiling

Microarray profiling of HCT116 cells stably silenced for GSTP1 was performed using the Illumina® HumanHT-12 v4 Expression BeadChip arrays (Wellcome Trust, University of Edinburgh). cRNA (750ng) from biological triplicates of HCT116 untreated, HCT116 GSTP1 shRNA 1, HCT116 GSTP1 shRNA 2 and HCT116 control plasmid was used in the array. Data from the array was analysed using Bioconductor 2.2 and normalised using quantile normalisation (robust multi-array average, RMA). The data was log2 transformed, and differential gene expression was examined between comparison groups using an empirical Bayes *t* test and corrected using Benjamini Hochberg correction. Probes exhibiting a p value of <0.05 were considered to be differentially expressed and used for enrichment analysis using Metacore pathway (Genego).

2.13 Biochemical assays

2.13.1 Biotinylation of glutathione ethyl ester (BioGEE)

BioGEE is a cell-permeable glutathione analogue which is transiently incorporated into proteins under conditions of oxidative stress and can therefore be used as marker of protein S-glutathionylation. Biotinylation of glutathione ethyl ester was prepared as previously described (Sullivan et al., 2000). 25mM sulfo-LC-NHS-biotin (Pierce) was added to 25mM glutathione ethyl ester (Sigma) and dissolved in 3ml of 100mM $NaHCO_3$ pH 8.5. The pH of the solution was adjusted to 7.2 with NaOH and incubated at room temperature for 2 hours. After 2 hours, 300µl of 1M Tris pH 7.2 was added to the solution to quench remaining biotinylation reagent and left to incubate overnight at 4°C before stored at -20°C.

For detection of protein S-glutathionylation *in vitro*, cells were incubated with 0.4mM BioGEE for 1 hour. Cells were then washed in ice cold PBS and harvested in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Igepal-630, 5mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 25mM N-ethylmaleimide. Biotinylated proteins were analysed as neat lysates by immunoblotting or purified using streptavidin-coupled Dynabeads® (Invitrogen) prior to immunoblotting.

2.13.2 Co-immunoprecipitation of proteins

For immunoprecipitation of biotinylated proteins, 40µl of streptavidin-coated Dynabeads® were washed 3 times in RIPA lysis buffer before incubated with 0.25-0.5µg/µl of protein lysate at 4°C for 1 hour under gentle rotation. Beads were separated from unbound protein lysates using a DynaMag™ magnet (Invitrogen) and were washed 4 times in RIPA wash

buffer. Biotinylated proteins were separated from streptavidin beads by heating the samples at 95°C for 5 min in 1x LDS Sample buffer (Invitrogen) in non-reducing conditions.

For the immunoprecipitation of GFP-tagged GSTP1 constructs, cells were lysed in NP40 buffer (10mM Tris-HCl pH 7.5, 150mM NaCl, 0.5mM EDTA, 0.5% NP40) and left on ice for 30 minutes, with extensive pipetting every 10 minutes. Lysates were centrifuged for 10 minutes at 13,000xg at 4°C and the supernatant removed. Protein lysate (2.5mg/ml) was incubated with 25µl GFP-Trap® -M beads (Chromotek) and incubated for 2 hours at 4°C under gentle rotation. Beads were separated from unbound protein lysates using a DynaMag™ magnet and were washed 4 times in NP-40 wash buffer. Immunocomplexes were separated from the beads by boiling in 1xLDS sample buffer for 10 minutes in reducing conditions.

2.13.3 Precipitation of proteins

Protein lysates were mixed with 4 volumes of ice cold acetonitrile and incubated on ice for 30 minutes. Lysates were centrifuged at 12,000rpm for 15 minutes at 4°C. Pellets were washed with fresh acetonitrile before centrifuged at 12,000rpm for 5 minutes at 4°C. Pellets were allowed to dry before resuspended in a suitable buffer solution for subsequent downstream applications.

2.13.4 Determination of protein concentration

The protein concentration of biological samples was determined spectrophotometrically using the Bradford Protein Assay (Bradford, 1976). For all assays, a working solution of Bradford Reagent (BioRad) was diluted 1:5 in water. Standards were prepared in duplicate containing

1ml of 0, 2, 4, 6, 8µg/ml BSA in Bradford Reagent. Protein lysates from samples were diluted in Bradford Reagent to a final volume of 1ml. Samples were mixed and incubated at room temperature for 5 minutes. The absorbance of each sample was measured at 590nm and the concentration in each sample was calculated against the standards.

2.13.5 Measurement of reduced and disulphide glutathione

Levels of total and reduced glutathione (GSH) were determined using a modification of the Tietze protocol (Tietze, 1969) as described by Rahman et al (Rahman et al., 2006). Levels of glutathione disulphide (GSSG) were determined using a modification of the Griffith protocol (Griffith, 1980). All steps were performed on ice unless stated otherwise. Glutathione levels were determined spectrophotometrically, using the sulfhydryl reagent 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB, also referred to as Ellman's reagent). DTNB oxidises GSH, forming GSSG and a TNB chromophore which can be measured at 412nm. The rate of TNB formation is proportional to the sum of GSH and GSSG present. Levels of GSSG are determined using GSSG reductase and monitoring NADPH spectrophotometrically. The levels of GSSG can be measured as described for GSH after treatment with 2-vinylpyridine, which covalently binds GSH and not GSSG (Rahman et al., 2006).

To determine the levels of GSH/GSSG *in vivo,* mouse liver samples were harvested and washed in ice cold PBS. 0.25M sucrose (2ml) was then added to approximately 100mg of tissue and homogenised; 500µl of homogenate was retained for protein determination. 2x extraction buffer (1.5ml; 0.2M potassium phosphate buffer pH 7.5, 10mM EDTA, 10% meta-phosphoric acid, 1.2% 5-sulfosalicyclic acid, 0.2% Triton X-100, 0.2% NP-40) was added to the remaining homogenate. Acidification and deproteinization of biological samples is

necessary to prevent oxidation of GSH and to inhibit the activity of γ-glutamyl transpeptidase which catalyses the transfer of glutathione to various molecules resulting in a loss of both GSH and GSSG. Homogenates were centrifuged at 3,000xg for 4 minutes at 4°C and 500µl of the aqueous upper layer was neutralised with 1ml of 1M Tris (pH 7.5) and used to determine GSH/GSSG levels.

To determine the levels of glutathione *in vitro,* cells were trypsinised and pelleted at 3,000xg for 4 minutes at 4°C. Pellets were washed in ice cold PBS, centrifuged and resuspended in 250µl 0.25M sucrose. An aliquot (50µl) of cell suspension was removed and used to determine protein concentration. Extraction buffer (excluding metaphosphoric acid) was added (200µl) to the sucrose suspension and centrifuged at 3,000xg for 4 minutes at 4°C. The supernatant was removed and used to determine GSH/GSSG levels.

Stock solutions of DTNB (4mg per 6ml), glutathione reductase (20 units per 6ml) and ß-NADPH (4mg/6ml) were prepared in KPE buffer (0.1M potassium phosphate buffer supplemented with 5mM EDTA). For determination of total and reduced levels of GSH, samples were diluted (1:20 for tissues, 1:5 for cells) and 20µl was dispensed in triplicate into a 96 well plate. GSH standards were prepared at 26.4, 13.2, 6.6, 3.3, 1.65, 0.825 and 0.4125µM in standard buffer (0.25M sucrose, 2x extraction buffer, 1M Tris (pH 7.5)). 20µl of each standard was dispensed in triplicate into a 96 well plate. DTNB and glutathione reductase (60µl of each) were added to each well and left for 2 minutes at room temperature. ß-NADPH (60µl) was added after 2 minutes and the velocity (mOD/min) of the reaction at 412nm was calculated. The increment in concentration of GSH was calculated and measured against the standard concentrations to determine total GSH concentration in the sample.

For determination of GSSG, 6µl of 2-vinylpyridine (diluted 1:10 in KPE buffer) was added to 300µl of homogenate and left at room temperature for 1 hour. Triethanolamine (18µl; diluted 1:6 in KPE) was added and left for 10 minutes in order to neutralise excess 2-vinylpyridine. Determination of GSSG was as described for GSH, with the following changes; 2% 1:10 2-vinylpyridine and 6% 1:6 triethanolamine is added to the standard buffer, samples are measured against GSSG standards at the concentrations described for GSH and samples are not diluted prior to analysis.

2.13.6 Measurement of mitochondrial respiration

Oxygen consumption and levels of glycolysis were determined using the XF24 Analyser (Seahorse Biosciences). Cells were seeded at 20,000 cells per well in 100µl media into a XF 24 TC well plate and incubated at 37°C/5% CO_2 for 1 hour to allow cells to adhere. DMEM or McCoy's 5A Medium (150µl) was added to each well and the cells were incubated overnight at 37°C/5% CO_2. At the same time, 1ml XF Calibrant solution, pH 7.4, was added to each well of a Seahorse Bioscience 24-well utility plate, the XF Sensor Cartridge placed on top and incubated at 37°C in a non-carbon dioxide incubator. The following morning, cells were gently washed in cell media before incubated in 825µl of unbuffered DMEM (8.3g/L DMEM base, 32mM NaCl, 25mM glucose, 1mM sodium pyruvate, 2mM GlutaMax-1, 42µM Phenol red, pH 7.4) for 1 hour at 37°C in a non-carbon dioxide incubator. During this period, 100µM of 2,4-dinitrophenol (DNP) dissolved in 75µl unbuffered media was added to one port of each well of the XF Sensor Cartridge and calibrated inside the XF Analyser. The cell plate was loaded onto the Analyser, mixed for 2 minutes, paused for 10 minutes followed by 5 cycles of mixing for 3 minutes, paused for 2 minutes and measured for 2.5 minutes. DNP

was then injected and the plate went through 2 cycles of mixing for 3 minutes, paused for 2 minutes and measured for 2.5 minutes.

2.13.7 Isolation of cytosol from mouse tissue

Cytosolic fractions were prepared from tissues as previously described with modifications (McLellan and Hayes, 1987). Tissue (100-200mg) was homogenised in 3 volumes of potassium chloride phosphate buffer (0.1M potassium phosphate pH 7.4, 0.1mM EDTA, 150mM potassium chloride, protease inhibitor (Roche)) and centrifuged at 11,000xg for 20 minutes at 4°C. The supernatant was removed and centrifuged at 100,000xg for 80 minutes at 4°C. The supernatant (cytosolic fraction) was removed and the pellet (micosomal fraction) was resuspended in sucrose buffer (0.25M sucrose in potassium chloride phosphate buffer supplemented with protease inhibitor (Roche)) and stored at -80°C.

2.13.8 Isolation of mitochondria from HCT116 cells

Mitochondria were isolated from cells using the Mitochondrial Isolation Kit for Cultured Cells (Pierce). $1x10^6$ HCT116 cells were seeded onto 10cm culture dishes and transfected with 10nM of siRNA targeted for GSTP1, a non-targeting control siRNA or untransfected as described previously. Cells were trypsinised 72 hours later and centrifuged at 800xg for 5 minutes and homogenised using a Dounce homogeniser. Mitochondria were isolated using differential centrifugation according to the manufacturer's protocol and stored at -80°C.

2.13.9 Immunoblotting

Immunoblotting was performed as previously described (Henderson and Wolf, 1992) with modifications. Protein lysates from cells and tissues were prepared at 0.5-2µg/µl in a sample buffer containing 1x LDS loading buffer (Invitrogen) and 10% ß-mercaptoethanol. For samples run under non-reducing conditions, ß-mercaptoethanol was omitted. Samples were boiled at 95°C for 5 minutes prior to loading onto gels. Protein (5-30µg) was resolved on 10-12% SDS-polyacrylamide gels at 100mV for 1.5-3 hours depending on the size of the protein of interest. Proteins were transferred onto Protean nitrocellulose membranes at 100mV for 80 minutes and visualised using Ponceau S solution (Sigma). Membranes were briefly washed in TBST buffer (50mM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.9) before blocked in 5-10% non-fat dry milk in TBST for 1 hour at room temperature. For immunoblotting of phospho-antibodies and biotinylated proteins, membranes were blocked in 5% BSA in place of milk for 1 hour at room temperature. Milk contains casein, a phospho-protein, causing high background with phospho-antibodies and due to the presence of endogenous biotin in non-fat dry milk, it should not be used for biotinylated proteins. Membranes were washed 3 times in TBST before incubated with a primary antibody (Appendices I) overnight at 4°C. Antibodies were prepared in 5-10% non-fat dry milk in TBST. Phospho-antibodies were prepared in 5% BSA in TBST. Membranes were washed 3 times in TBST and incubated with secondary antibody prepared in 5% non-fat dry milk for 1 hour at room temperature. Membranes were washed 4-5 times in TBST before visualised using a chemiluminescent kit (ECL, Millipore) and Konica Minolta autoradiographic film.

2.13.10 Two-dimensional gel electrophoresis

Biotinylated proteins from HCT116 cells were analysed by two-dimensional gel electrophoresis as previously described with modifications (Berkelman and Stenstedt, 1998). Protein lysate (100µg) was precipitated using acetonitrile and pellets were resuspended in 100µl of isoelectric focusing (IEF) buffer containing 7M urea, 2M thiourea, 4% CHAPS, 12µl/ml Destreak, 5µl/ml Bio-Lyte ampholytes and 0.002% bromophenol blue. Sample loading was performed by overnight in-gel rehydration of Immobilised pH Gradient (IPG) ReadyStrips™ pH3-10 NL, pH4-7 or pH5-8. 20µg of sample was used for silver stain analysis and 70µg of sample used for Western blotting. IEF was performed at 20°C using a Protean IEF Cell (BioRad) according to the following parameters; 250V for 15minutes, 4,000V for 2 hours, 4,000V for 20,000Vhours, hold at 500V. Voltage ramping was linear and the current limit did not exceed 50µA/strip.

Immobilized pH gradient (IPG) strips were equilibrated in buffer (EQ buffer) containing 0.37M Tris, 6M urea, 2% SDS, 30% glycerol pH 8.8. For analysis of proteins using silver staining, strips were washed 3x 5 minutes in EQ buffer containing 130mM DTT, followed by washing 3x 5 minutes in EQ buffer containing 135mM iodoacetamide. For analysis of biotinylated proteins using western blotting, DTT was omitted from the buffer. IPG strips were resolved on 10-12% SDS-polyacrylamide gels at 80mV for 2-3 hours and analysed by silver staining or western blotting.

2.13.11 Silver staining

Silver staining of SDS-polyacrylamide gels was performed using the SilverQuest staining kit (Invitrogen). Gels were briefly washed in ultrapure water before fixed overnight in a solution

containing 40% ethanol, 10% acetic acid and 50% ultrapure water. Gels were then stained according to the manufacturer's instructions.

2.14 Statistical analysis

All results are expressed as mean ± standard deviation. ANOVA, Student's *t*-test and the Mann-Whitney test are used where parametric and non-parametric analyses are indicated. Non-normality was determined using the Shapiro-Wilk test. All statistical analysis was performed using SPSS statistical software (University of Dundee).

3. Chapter 3: Non-catalytic functions of GSTP1 in mediating resistance to acetaminophen-induced hepatotoxicity

Introduction

GSTP1 plays an important role in cell physiology, the precise mechanism(s) of which are yet to be fully characterised. There are a growing number of *in vitro* studies highlighting functions of GSTP1 independent of its catalytic properties, yet little *in vivo* evidence to corroborate these findings. This chapter will begin to assess some of the non-catalytic properties of GSTP1 using a novel transgenic mouse model in which the activity of mouse GSTP1 (mGSTP1) has been made catalytically redundant. Preliminary studies will examine basal function of GSTP1 and assess whether its catalytic activity contributes to the hepatotoxic effects of acetaminophen.

3.1 *In vivo* characterisation of GSTP1 through the use of *Gstp1/2*$^{-/-}$ mice

Transgenic models provide a powerful tool in characterising the functions of a gene *in vivo*. Mice have a more comparable GST isozyme composition to humans than other laboratory animals such as the rat (Gupta et al., 1990) which makes them ideal candidates in modelling GSTs in transgenic research. A number of GST classes have been deleted *in vivo* which has led to the identification of several interesting phenotypes. For a thorough overview on the use of transgenic models in GST research please refer to a recent review by Henderson and Wolf, 2011 (Henderson and Wolf, 2011). In contrast to most other organisms, mice have 2 *Gstp* genes, *Gstp1* and *Gstp2* which are located on chromosome 19 and lie in tandem 2.5kb

apart. GSTP2 is catalytically weaker than GSTP1 and transcribed at a lower level despite differing by six amino acids and sharing 97% sequence identity (Bammler et al., 1995). The genes encoding GSTP1 and GSTP2 have been knocked out, resulting in *Gstp1* null (*Gstp1*$^{-/-}$) and *Gstp1/2* null (*Gstp1/2*$^{-/-}$) mice bred, initially, on a 129xMF1 background (Henderson et al., 1998b). To date, the function of GSTP1 has only been assessed in comparison to *Gstp1/2*$^{-/-}$ mice. The *Gstp1* gene and exons 5-7 of the *Gstp2* gene have been replaced by an IRES-ßGEO element, the targeting construct shown in Figure 3.1. GSTP2 contains very little catalytic activity in its native form and truncation of the *Gstp1* gene shows no catalytic activity towards CDNB (Henderson et al., 1998a). No transcript or protein expression of the truncated *Gstp2* gene could be identified as determined by Western or Northern Blotting (Henderson et al., unpublished). *Gstp1/2*$^{-/-}$ null mice are viable, phenotypically healthy with no apparent state of stress or illness and have no apparent defect in reproductive capabilities, the only apparent difference being a greater body weight and lung size compared to their wild-type counterparts (Henderson et al., 1998a), the reason for which remains unclear. With the complete deletion of a single class of GST, one might expect an adaptive mechanism to compensate through increased expression of other GST classes. Whilst other GST knock out models have observed some form of compensatory changes (Fernandez-Canon et al., 2002) this is not apparent at a protein level in *Gstp1/2*$^{-/-}$ mice (Henderson et al., 1998b, Kitteringham et al., 2003). Despite a lack of protein induction, it has been observed that there is significant increase in other GST mRNA levels and other antioxidant response genes upon deletion of GSTP1 (Henderson et al., 2005, Elsby et al., 2003).

Like other GST knockout mouse models, *Gstp1/2*$^{-/-}$ mice only appear to exhibit differences in phenotype in response to chemical or genetic stress. Upon exposure to chemical agents, the deletion of GSTP1 can have detrimental effects *in vivo*, often, but not exclusively, associated

with an ability to suppress inflammatory responses. In a study by Henderson et al. *Gstp1/2*$^{-/-}$ mice were treated with 7,12-dimethylbenz anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), used respectively as skin tumorigenic inducing and promoting agents. The authors found that *Gstp1/2*$^{-/-}$ mice developed a significantly higher number of papillomas compared to wild-type animals; an average of 9.94 papillomas per animal compared to 2.89 in wild-type mice ($P<0.001$). Later studies suggested that the role of GSTP1 in supressing inflammation may account for these observations, as *Gstp1/2*$^{-/-}$mice crossed onto mice harbouring H-ras mutations in the skin (Tg.Ac mice) showed increased nitrotyrosine formation and upregulation of a number of inflammatory genes compared with their wild-type counterparts when treated with TPA (Henderson et al., 2011). This has considerable implications in humans, where GSTP1 expression and catalytic activity is far higher in female skin and may lead to sex differences in the metabolism of polycyclic aromatic hydrocarbons (PAHs) depending on the level of exposure (Singhal et al., 1993).

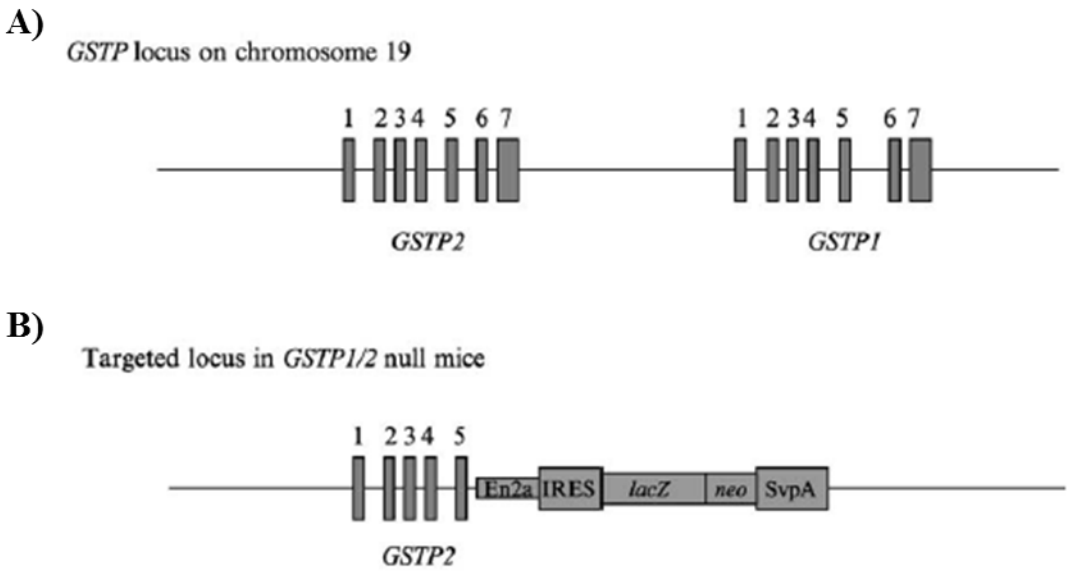


Figure 3.1. Targeting strategy for the deletion of the murine *Gstp* cluster, taken, with permission, from Henderson et al, 2005.

A) Target gene cluster **B)** Targeted deletion of *Gstp1 and Gstp2*; En2a, splice acceptor site; IRES, internal ribosome entry site; lacZ, β-galactosidase reporter; pA, simian virus 40 polyadenylation site.

GSTP1 expression also has a profound effect in the protection against pulmonary carcinogens. In the human lung, GSTP1 is the most prominent GST but this is not the case in rodents, where the expression of GST Mu and Alpha classes are higher. It is clear however, that GSTP1 expression is an important factor in lung pathophysiology. Ritchie et al demonstrated that *Gstp1/2*$^{-/-}$ mice treated with the PAH agents benzo[a]pyrene (BaP), 3-methylcholanthrene (3-MC) and the carbamate urethane, developed more papillomas on the lung surface than wild-type mice and had increased BaP DNA adduct formation (Ritchie et al., 2007). GSTP1 is known to be involved in the metabolism of different PAHs (Sundberg et al., 1998, Hu et al., 1997, Whyatt et al., 2000) and therefore may explain the difference in sensitivity towards these agents in *Gstp1/2*$^{-/-}$ mice. Pulmonary expression of GSTP1 also

appears to attenuate inflammatory responses in mice. Zhou et al demonstrated that *Gstp1/2*$^{-/-}$ mice challenged with ovalbumin to induce airway inflammation show increased eosinophilia and goblet cell hyperplasia compared to wild-type mice, while Schroer et al, showed increased oxidative stress in *Gstp1/2*$^{-/-}$ mice subjected to an allergen challenge (Zhou et al., 2008, Schroer et al., 2011). A potential mechanism of GSTP1 cytoprotection in pulmonary disease was highlighted by Schroer et al as GSTP1 was found to be downregulated in children with asthma.

These studies form part of a larger body of evidence which suggests that suppression of inflammatory signalling may be one mechanism by which GSTP1 functions *in vivo*. *Gstp1/2*$^{-/-}$ mice crossed onto mice heterozygous for mutations in the adenomous polyposis coli (*Apc*) gene have a higher incidence and multiplicity of colon adenomas than their wild-type counterparts, and demonstrate an induction of a number of inflammatory genes and formation of nitrotyrosine adducts (Ritchie et al., 2009). The *Apc*^{min} mouse model carries a missense mutation at codon 850 of the *Apc* gene, resulting in small intestinal adenoma development through activation of Wnt signalling driven by ß-catenin (Su et al., 1992) and therefore bypasses practical and metabolic problems associated with chemically induced carcinogenesis. By crossing *Gstp1/2*$^{-/-}$ mice onto *Apc*^{min} mice, the authors managed to define a phenotype of GSTP1 which is independent of chemical stress and metabolism. The mechanism behind the suppression of inflammatory response remains unclear but may be related to the regulation of the mitogen-activated protein kinases (MAPKs). A model of GSTP1 mediated cell regulation was proposed by Adler et al, when, using mouse embryonic fibroblasts (MEFs), the group identified that GSTP1 binds to c-Jun N-terminal kinase (JNK) preventing downstream phosphorylation of its target substrates (Adler et al., 1999). JNK is an upstream modulator of the transcription factor AP-1 and is intrinsically controlled to

phosphorylate c-Jun in the presence of stressors such as ionising and UV irradiation, heat shock, growth factors, inflammatory cytokines or oxidative stress (Westwick et al., 1994, Cano et al., 1994, Hibi et al., 1993). Under basal conditions, GSTP1 binds to the C-terminus of JNK in its monomeric form, preventing phosphorylation of c-Jun by JNK (Wang et al., 2001b). In times of oxidative stress, the GSTP1 monomer oligomerizes and dissociates from JNK, which becomes phosphorylated and activates the MAPK pathway and subsequent stress responses. Studies using MEFs and 3T3 fibroblast cells suggests that the JNK2 isoform contributes to JNK stability whereas the JNK1 isoform activates downstream activity (Sabapathy et al., 2004). Constitutive JNK signalling may then lead to the recycling of GSTP1 transcription as the phosphorylated c-Jun forms a complex with other transcription factors such as c-Fos to form the AP-1 complex which binds to the AP-1 promoter on the *GSTP1* gene (Moffat et al., 1994). Subsequent *in vivo* work has demonstrated that *Gstp1/2*$^{-/-}$ mice bred on a 129xMF1 background express higher basal levels of JNK in liver and lungs and also express increased levels of an AP-1 target antioxidant gene, Haem Oxygenase-1 (HO-1) and the ARE regulated phase II gene, UDP-glucuronosyltransferase 1A6 (Elsby et al., 2003). *Gstp1/2*$^{-/-}$ mice administered cyclophosphamide show a larger increase in bladder toxicity and acrolein adduct formation compared to wild-type mice and demonstrate increased JNK activity, in part due to an inability to metabolise acrolein in the absence of GSTP1 (Conklin et al., 2009). *Gstp1/2*$^{-/-}$ mice have been found to have higher levels of myeloproliferation through increased levels of JNK and signal transducer and activator of transcription (STAT) molecules, particularly in bone marrow cells, and have higher levels of circulating leukocytes than their wild-type counterparts (Gate et al., 2004).

Despite extensive research into this mechanism, a lack of consistency and detail across *in vitro* and *in vivo* models demonstrates that GSTP1 regulation of JNK signalling cannot be a

universal mechanism to explain GSTP1-associated phenotypes (discussed further in 'Results Chapter 4' and 'Discussion'). As discussed below, *Gstp1/2*$^{-/-}$ mice are resistant to the hepatotoxic effects of acetaminophen and show increased resistance to the nephrotoxic effects of cisplatin (Townsend et al., 2008b). The latter phenotype can be rationalised through increased production of cysteine S-conjugate β-lyase in the proximal tubule, as a result of increased cisplatin conjugation, which results in the formation of reactive thiols. However, this phenotype is not translated clinically or *in vitro*, as GSTP1 has been shown to attenuate cisplatin efficacy (Peklak-Scott et al., 2008) while the use of a GSTP1 inhibitor, ethacrynic acid, increases the sensitivity of tumour cells to cisplatin (Wang et al., 2007). The role of GSTP1 in mediating cytoprotection appears more complex than its function in enzymology but cannot be solely explained through the regulation of JNK.

3.2 Acetaminophen

Initially identified as the metabolite derived from acetanilide and phenacetin (Brodie and Axelrod, 1948a, Brodie and Axelrod, 1948b) acetaminophen has been widely used since the 1950s as an analgesic and antipyretic compound. At therapeutic doses (500mg) acetaminophen is well tolerated but can cause hepatic necrosis and acute liver failure at higher doses. The metabolism of acetaminophen is well established with around 80-90% of the parent compound typically conjugated with sulphate or glucoronide and excreted in the urine (Forrest et al., 1982, Clements et al., 1984). Around 5% of acetaminophen is oxidised in a cytochrome P450 directed manner, with CYP2E1, CYP3A4 and CYP1A2 isoforms catalysing the majority of CYP-mediated reactions (Raucy et al., 1989, Patten et al., 1993). Hepatotoxicity as a result of acetaminophen overdose was first established in 1966 (Davidson and Eastham, 1966), the toxicity of which is directly linked to increased production of a reactive metabolite from CYP-mediated reactions, N-acetyl-*p*-benzoquinone imine (NAPQI).

At non-toxic doses, NAPQI is conjugated to the tripeptide glutathione, to form 3-glutathion-*S*-yl-acetaminophen (Hinson et al., 1982), which can be catalysed by GSTP1 or non-enzymatically conjugated (Coles et al., 1988). At toxic doses, glucoronidation and sulfation pathways become saturated, leading to increased NAPQI production and depletion of hepatic glutathione levels. The electrophilic NAPQI can arylate a number of thiol containing peptides and proteins (Bartolone et al., 1987) and activate stress response genes, leading to DNA damage (Ray et al., 1993), increased ALT/AST production (Pumford et al., 1989), mitochondrial damage (Burcham and Harman, 1991, Weis et al., 1992) and changes in calcium homeostasis (Moore et al., 1985, Ray et al., 1993), resulting in hepatic necrosis. Inhibition of activation of these pathways or administration of cysteine containing compounds such as N-acetyl-cysteine can be used as effective treatment against acetaminophen overdose (Gunawan et al., 2006, Smilkstein et al., 1988).

Mice are particularly sensitive to the hepatotoxic effects of acetaminophen compared to other laboratory animals, in part due to a rapid depletion of hepatic glutathione after acetaminophen treatment (Green et al., 1984). The sensitivity of mice towards acetaminophen appears to be strain specific as Harrill et al demonstrated a range of ALT serum responses and differential changes in gene expression after a single dose of acetaminophen from a panel of 36 inbred mouse strains (Harrill et al., 2009a). Using this model, Harrill et al went on to perform a whole-genome association analysis to identify that polymorphisms in the *Cd44* gene in mice correlated well with tissue necrosis, and later identified that polymorphisms in the orthologous human gene, *CD44*, show comparable sensitivity traits to acetaminophen in a human cohort (Harrill et al., 2009b). Despite its functional role in catalysing glutathione conjugation of NAPQI, there is little evidence to suggest that polymorphisms in the *GSTP1* gene may confer differences in susceptibility to acetaminophen treatment. This is not

surprising, as slight changes in catalytic activity due to polymorphic variations may have little effect on the conjugation of glutathione to NAPQI if non-enzymatic reactions can also take place in acetaminophen overdose. However, there is some evidence to suggest that asthma induced by prenatal exposure to acetaminophen may be influenced by polymorphisms in *GSTP1* (Perzanowski et al., 2010).

The role of GSTP1 in mediating acetaminophen metabolism becomes more complex as, paradoxically, *Gstp1/2*$^{-/-}$ mice appear to be more resistant to the hepatotoxic effects of acetaminophen than their wild-type counterparts. Using acetaminophen as a model compound for hepatotoxicity, Henderson et al, demonstrated that after a single dose of acetaminophen, *Gstp1/2*$^{-/-}$ mice had lower levels of plasma ALT and very little hepatic necrosis compared to their wild-type counterparts, which showed significant increases in ALT and substantial hepatic necrosis at 24 and 48 hours after dosing (Henderson et al., 2000). The difference in sensitivity was attributed to increased hepatic glutathione regeneration after acetaminophen treatment in *Gstp1/2*$^{-/-}$ mice, as no difference was observed in the pharmacokinetic metabolism or conjugation of the parent compound. This suggests that covalent binding of acetaminophen may not be the only mechanism of toxicity and that regulation of other cellular pathways involved in acetaminophen induced hepatotoxicity may be mediated by GSTP1. Therefore, *in vivo* modelling of a catalytically inactive GSTP1 protein may aid in elucidating these pathways associated with hepatotoxicity in mice.

3.3 Structure and Kinetics of GSTP1 - Identification of Tyr7 as target for non-catalytic mouse model

Reinemer et al first identified the three dimensional structure of GSTP1 using cytosolic extracts isolated from pig lung and showed that GSTP1 is composed of a dimer with two identical subunits of 23 kDa (Reinemer et al., 1991). The three-dimensional structures have since been characterised for human (Reinemer et al., 1992) (Figure 3.2) and mouse (Parraga et al., 1998) GSTP1. The ability of GSTP1 to dimerize appears critical for the stability of its tertiary structure (Erhardt and Dirr, 1995). Similarly to other cytosolic GSTs, dimerization is facilitated through ionic, hydrogen bonding and a hydrophobic 'lock and key' motif between its subunits, of which Tyr50 appears to be a key residue in mediating hydrophobic interactions. Although *in vitro* experiments have demonstrated heterodimerization between GSTP1 and GSTM1 (Pettigrew and Colman, 2001), it is unlikely that this occurs *in vivo*. Despite only around 23 residues being conserved amongst cytosolic GST classes, GSTP1 is structurally similar to the Alpha and Mu class of GSTs but differs in its C-terminal region, possessing a highly exposed electrophilic binding site that expresses both hydrophilic and hydrophobic interactions (Ji et al., 1997). There is evidence to suggest that GSTP1 can exist as a stable monomer and in its reduced form can interact with several proteins such as JNK and PrdxVI (Monaco et al., 1999, Manevich et al., 2004, Adler et al., 1999). In HaCaT cells, increased cell survival and inhibition of JNK phosphorylation is associated with stabilisation of GSTP1 monomers by the Human Papillomavirus-16 E7 protein (Mileo et al., 2009). However, there is contrasting evidence to show if monomeric GSTP1 has any catalytic function with some reports suggesting a lack of catalytic functionality of GSTP1 monomers (Abdalla et al., 2002), whilst others suggesting dimerization stabilises the catalytic function but is not required for catalysis (Huang et al., 2008a).

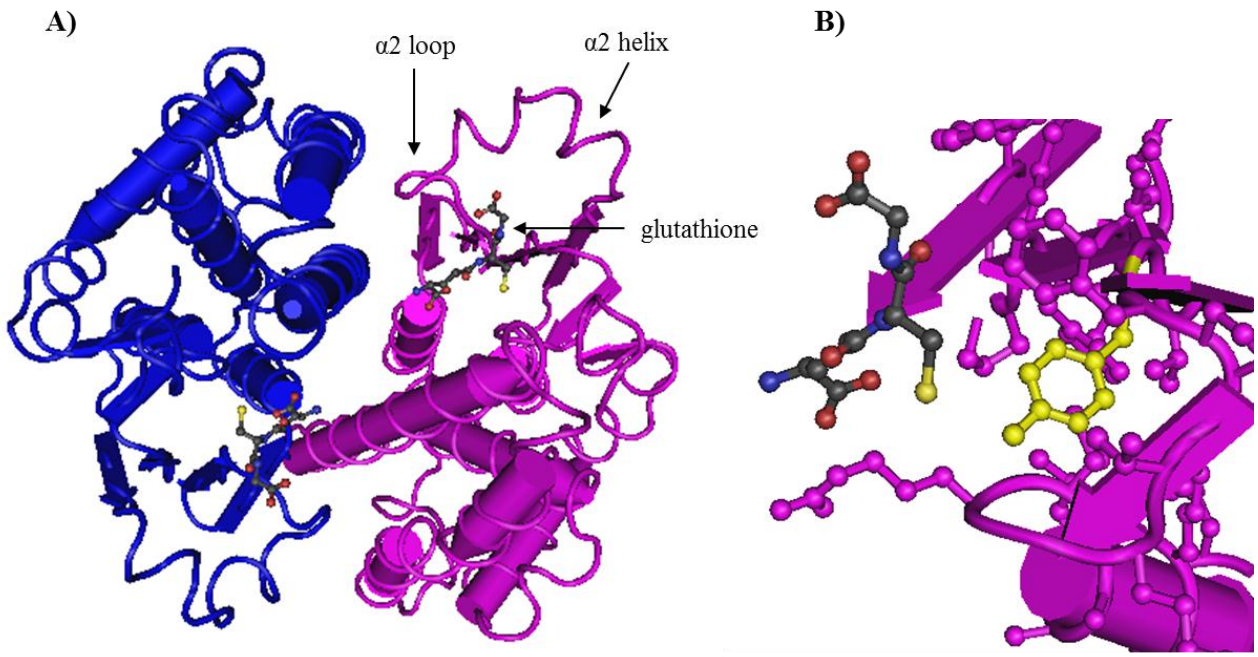


Figure 3.2. Three dimensional structure of hGSTP1-1 in complex with glutathione.

A) Dimeric structure of human GSTP1-1. Helix α2 is highly flexible within GSTP1-1 and can modulate glutathione binding and catalysis (Oakley et al., 1997). **B)** Position of tyrosine 7 (highlighted in yellow) in relation to glutathione within the N-terminal domain of GSTP1-1.

Activation of glutathione by GSTP1 is mediated through a tyrosine (Tyr7) present within its active site (Reinemer et al., 1991) and is conserved in all cytosolic GSTs (Mannervik et al., 1985a). Kinetic studies using recombinant human GSTP1 demonstrated that Tyr7 contributes predominantly to the catalysis of substrates rather than their binding as mutational studies in which tyrosine is substituted to a phenylalanine demonstrates large decreases in catalytic activity, yet very little change in substrate affinity (Kolm et al., 1992, Cesareo et al., 2005). Its catalytic function is thought to be mediated through protonation of a phenolic hydroxyl group present in the tyrosine molecule (Kolm et al., 1992) which stabilises the glutathione anion (GS^-) by activating its sulphur group through hydrogen bonding (Stenberg et al., 1991).

Although structurally similar, phenylalanine lacks this phenolic hydroxyl group and therefore catalysis cannot occur. Later studies have argued against the tyrosinate hypothesis as the proton acceptor and suggested that a water molecule found within the G-site accepts and transfers the thiol proton (Parraga et al., 1998).

A number of key residues involved in GSTP1-mediated catalysis and substrate binding have been identified and are highlighted in Table 4. Mutation of residues within the lock and key motif of GSTP1 (Tyr50) produces large decreases in catalytic activity (K_{cat}) towards a number of substrates (Hegazy et al., 2004), but the inability of GSTP1 to dimerize accurately as a result of the mutations deems the lock and key motif an unsuitable target for *in vivo* modelling of the non-catalytic properties of GSTP1. Other substitutions affecting dimerization were similarly disregarded as potential targets. In 1995, Bammler et al, neatly identified that the differences in catalytic activity between mouse GSTP1 and the non-catalytic protein GSTP2, was largely dependent on 2 key amino acid substitutions, located within the H-binding site (Val10) and N-terminal domain (Arg11) of GSTP1 (Bammler et al., 1995). Site-directed mutagenesis of these sites resulted in large decreases in catalytic activity, with Val10Ser demonstrating an 80% loss in activity while Arg11Pro a 97% loss in activity. Although Val10Ser resulted in modest changes in affinity for glutathione and CDNB, Arg11Pro resulted in reduced affinity for both glutathione and CDNB and therefore was dismissed as a possible target for *in vivo* modelling.

The advantage for targeting a Tyr7 in the development of a non-catalytic GSTP1 mouse model is that as Tyr7 is crucial for the catalytic activity of GSTP1 but is not required for

substrate binding, mutation of Tyrosine to Phenylalanine may result in a protein which is devoid of catalytic activity yet retains its ability to bind glutathione and other substrates.

Element	**Mutation**	**Comments**	**Advantage as a mutation target**	**Disadvantage as a mutation target**	**Reference**
GSH catalysis / binding	GSTP1 (10 mutations)	Stable monomer. 10 mutations - L49Q, Y50E, L61Q, L63E, A87E, A88K, L89E, M92E, G96K, C102Q – loss of catalytic activity, retained affinity for several H-site compounds.	Stable and has complete loss of catalytic activity	Problems with dimerization and introduction of 10 mutations may be problematic.	(Abdalla et al., 2002)
	Tyr 7 (Y7F)	Tyr 7 important for catalysis. Large decrease in GSH Kcat. Moderate increase in GSH Km.	Largest decrease in catalytic activity	Key site in glutathionylation of proteins	(Kong et al., 1992) (Kolm et al., 1992) (Cesareo et al., 2005) (Townsend et al., 2008a)
	Tyr50 (Y50A)	Reduction in GSH Kcat but increase in GSH Km. Heterodimer of mutant and wild-type similarly reduces Kcat but only slight increase in Km.	Loss of catalytic activity in mutant and heterodimer. Role in GSH stability.	Loss of GSH affinity. Affects dimerization. Heterodimer not practical in vivo	(Hegazy et al., 2004)
Substrate binding	Val10 (V10G) (V10S)	Reduced catalytic activity towards EA (V10G) and CDNB (V10G, V10S), GSH Km unchanged	Large reduction in Kcat of substrates with hardly no loss	Only compounds tested are CDNB and EA – unsure of effect on other substrates	(Micaloni et al., 2000) (Bammler et al.,

		(V10G, V10S)	in GSH affinity		1995)
	Arg11 (R11P)	Decreased CDNB Kcat, increased CDNB Km. GSH Km increased.	Largest decrease in Kcat of all substrate mutations	Large loss of GSH affinity. Uncertain of effect on other substrates.	(Bammler et al., 1995)
	Tyr50 (Y50A)	Reduction in substrate and GSH Kcat but increase in Km towards CDNB (aromatic nucleophilic displacement) and PEITC (addition reaction).	Decrease in kcat for several types of reactions	Affects dimerization	(Hegazy et al., 2004)
	Val104 (V104G)	Decrease CDNB Kcat, increase GSH km.	Mutation has implications in human polymorphisms and in altered transformation of various compounds.	Not potential site to study non-catalytic mechanisms as decrease in Kcat is only moderate.	(Bammler et al., 1995) (Abel et al., 2004)
	Tyr108 (Y108F)	Reduced Kcat towards ethacrynic acid, no change in GSH Km. Reduced Kcat towards EPNP. Important site for catalysis and binding of epoxide substrates. Little effect on CDNB, DCNB, cumene hydroperoxide DTNB, 4NQO and Δ^5-androstene 3,17-dione.	Reduction in substrate kcat with minimal decrease in GSH affinity	Has little effect on a variety of compounds.	(LoBello et al., 1997) (Park et al., 2005)
Dimerization	Tyr50 (Y50A)	Key residue in lock-and-key motif. Not strongest contributor in dimerization of	A key residue with regards to GSH stability. Decrease in	Not strongest residue with regards to dimerization	(Hegazy et al., 2004)

		GSTP1-1 but important for thermal stability of dimer.	GSH and substrate kcat for several types of reactions		
	Arg70 (R70Q)	Mutation increases GSH Km compared to wild-type and affects dimer stability. No change for CDNB Km. Removing charge lowers enzymatic activity.	Minimal effect on substrate Km.	Large loss of GSH affinity and large effect on dimer stability.	(Huang et al., 2008b)
	Arg74 (R74Q)	Not determinant of GSH stability. Small increase towards GSH Km of R74Q mutant. No change for CDNB Km. Removing charge lowers enzymatic activity and diminishes dimerization.	Minimal effect on substrate Km.	Diminished dimerization will effect GSTP1 regulation in vivo	(Huang et al., 2008b)
	Asp90 (D90N)	Not determinant of GSH stability. Conserved in GSTP1. Mutation causes no change for CDNB Km. Removing charge lowers enzymatic activity and diminishes dimerization.	Minimal effect on substrate Km.	Diminished dimerization will effect GSTP1 regulation in vivo	(Huang et al., 2008b)
	Asp94 (D94N)	No change for CDNB Km. Marked increase in GSH Km. Removing charge lowers enzymatic activity and shifts	Minimal effect on substrate Km. Decreased enzymatic activity.	Large loss of GSH affinity and large effect on dimer stability.	(Huang et al., 2008b)

		equilibrium towards monomer.			

Table 4. Mutation of residues in GSTP1 that confer differences in kinetic and binding properties.

However, the consequence of this mutation in the regulation of cellular proteins is somewhat complex and difficult to predict. Whilst groups have demonstrated that the Y7F mutant is as efficient as wild-type GSTP1 in inhibiting high basal JNK activity and c-Jun-mediated transactivation (Adler et al., 1999, Townsend et al., 2008a), the Y7F mutant does not appear to form complexes with PrdxVI (Ralat et al., 2006) and has diminished ability to glutathionylate cellular proteins (Townsend et al., 2008a).

To ensure that a Y7F substitution would result in decreased catalytic activity in mouse GSTP1, recombinant mouse GSTP1 harbouring the wild-type or Y7F mutation was produced and used in kinetic studies against GSH and CDNB to determine catalytic rates and substrate affinity. Table 5 highlights the kinetic data of $GSTP1^{WT}$ and $GSTP1^{Y7F}$ recombinant proteins. $GSTP1^{Y7F}$ shows a marked decrease in its catalytic rate (V_{max}) against GSH and CDNB when compared against wild-type GSTP1. This correlates with previous reports that a Y7F mutation greatly reduces the catalytic activity of GSTP1. It also demonstrates a more marked reduction in catalytic activity than mutations associated with catalytic differences between GSTP1 and GSTP2 as observed by Bammler et al (Bammler et al., 1995). While the affinity of $GSTP1^{Y7F}$ for CDNB remains unchanged there is a slight increase in its affinity for GSH. However, the kinetic data show that a Y7F substitution would be a promising model to use in

the development of a non-catalytically functioning GSTP1 protein which retains its ability to bind to substrates.

Kinetic parameters[1]				
Protein	K_m^{GSH}	V_{max}^{GSH}	K_m^{CDNB}	V_{max}^{CDNB}
$GSTP1^{WT}$	0.65±0.1	397±20	0.55±0.1	447±39
$GSTP1^{Y7F}$	$0.31±0.05^{**}$	$4.4±0.17^{***}$	$0.67±0.1^{ns}$	$3.9±0.2^{***}$

[1] K_m:µM; V_{max}:$\Delta^{340nm/min/mg}$

Table 5. Apparent kinetic parameters of mouse $GSTP1^{WT}$ and $GSTP1^{Y7F}$ towards 1-chloro-2,4-dintrobenzene (CDNB) and glutathione (GSH).

Data are provided for the kinetic parameters of recombinant protein, where affinity (K_m) is shown as µM and the V_{max} as the rate of change at 340nm/min/mg protein. Data are provided as mean ± standard deviation (n=4) where ns, not statistically significant, ** *P* value <0.01, *** *P* value <0.001.

3.4 Generation and design of *Gstp1*^{Y7F} mouse model

The data generated from the recombinant studies demonstrate that targeting the Tyr7 residue would provide the best model for examining the non-catalytic function of GSTP1 *in vivo*. A mouse model was designed in which Y7F was constitutively knocked into exon 2 of the mouse *Gstp1* gene using a BAC transgene (Figure 3.3) as described in 'Materials and Methods'. Neomycin and puromycin resistance sites used for clonal selection have been flanked by FRT and F3 sites, respectively, which are then removed after Flp recombination to avoid any alteration of gene function (Scacheri et al., 2001). Despite the relatively low transcription and catalytic activity of GSTP2, there are some concerns that it may have a role

in cell regulation in a non-catalytic capacity. To circumvent any role GSTP2 may play in cell regulation and cytoprotection, loxP sites were introduced flanking exons 1 to 4 of the *Gstp2* gene, allowing for conditional deletion of *Gstp2* using Cre recombination. This would allow for a mouse model solely dependent on the non-catalytic properties of *Gstp1*. However, in order to control against the effects of *Gstp2* against their wild-type counterparts, all data presented here utilises *Gstp1*^{Y7F} mice harbouring a *Gstp2* gene.

3.5 Characterisation of *Gstp1*^{Y7F} mice

3.5.1 Protein expression

Gstp1^{Y7F} mice show no abnormal phenotype during development, suggesting that the catalytic function of GSTP1 is not essential for survival. Although some reports suggest *Gstp1/2*$^{-/-}$ mice bred on a 129xMF1 background develop a higher incidence of spontaneous tumours, particularly lung adenomas (Gate et al., 2005), we found no evidence for this in *Gstp1/2*$^{-/-}$ or *Gstp1*^{Y7F} mice bred on a C57Bl/6J background up to 18 weeks of age (data not shown). Despite reports of increased lung size in *Gstp1/2*$^{-/-}$ mice compared to wild-type mice bred on a 129xMF1 background (Henderson et al., 1998a), we found no evidence of this in either transgenic mouse model bred on a C57Bl/6J background. The only differences observed were sex-related, with female *Gstp1/2*$^{-/-}$ mice having a larger brain size than their male counterparts and female *Gstp1*^{Y7F} mice having a larger thymus than their male counterparts (Table 6). The differences in organ:weight ratios in this instance has not been examined further.

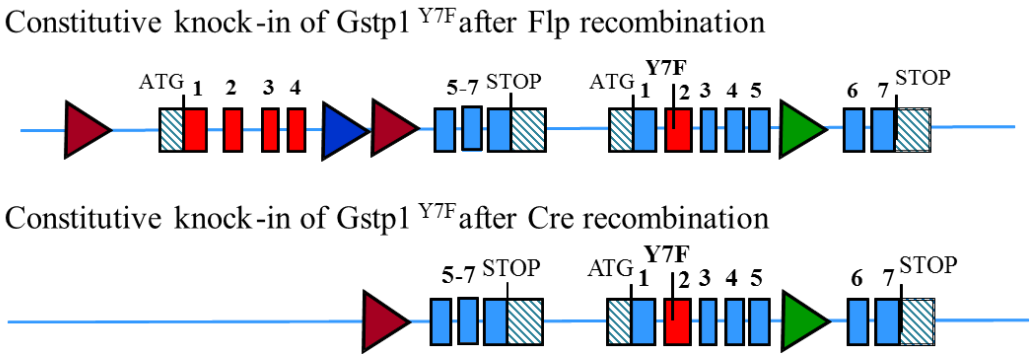


Figure 3.3. Targeting strategy for the generation of a GSTP1^{Y7F} mouse model.

The diagram shows the introduction of Y7F into exon 2 of the *Gstp1* gene which is downstream of the *Gstp2* gene. Exons 1-4 of *Gstp2* are flanked by LoxP sites (purple arrows) allowing conditional deletion of *Gstp2* using Cre recombination. Green arrow, FRT site. blue arrow, F3 site.

GSTP1 is expressed largely in the livers of mice, particularly in male mice (Henderson et al., 1998b). For this reason, male mice are the ideal model to use when examining the function of GSTP1 compared to a knockout system. The expression of GSTP1 from the livers of *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice were analysed by Western blotting (Figure 3.4). We can clearly observe the presence of GSTP1 in the livers of *Gstp1*^{WT} and *Gstp1*^{Y7F} mice and demonstrate that they are expressed at comparable levels, suggesting that GSTP1 is translated to the same extent in the liver of the knock-in mouse model as its wild-type counterpart. As predicted, no GSTP1 protein can be detected in *Gstp1/2*$^{-/-}$ mice. This demonstrates that the antibody used is specific for GSTP1, as it shows no cross-reactivity with other GST classes. There was no evidence to suggest that deletion or catalytically inactivating GSTP1 resulted in upregulation of other GST classes as a compensatory mechanism, an observation which correlates with previous reports (Kitteringham et al., 2003).

Organ	**Organ:weight ratios**					
	Male			**Female**		
	Gstp1^{WT}	*Gstp1/2*$^{-/-}$	*Gstp1*^{Y7F}	*Gstp1*^{WT}	*Gstp1/2*$^{-/-}$	*Gstp1*^{Y7F}
Bladder	0.09±0.024	0.12±0.004	0.12±0.05	0.06±0.03	0.09±0.004	0.11±0.07
Brain	1.66±0.13	1.78±0.09*	1.77±0.1	2.07±0.08	2.21±0.27*	2.15±0.16
Kidney	1.19±0.13	1.2±0.12	1.46±0.16	1.08±0.009	1.11±0.18	1.17±0.07
Liver	5.4±1.01	5.36±0.31	4.95±1.01	5.44±0.19	4.52±0.69	4.52±1.05
Lung	0.59±0.01	0.61±0.04	0.622±0.08	0.68±0.04	0.69±0.04	0.72±0.03
Spleen	0.36±0.15	0.3±0.02	0.38±0.17	0.35±0.02	0.42±0.1	0.42±0.04
Testes	0.64±0.07	0.8±0.19	0.58±0.12	n/a	n/a	n/a
Thymus	0.25±0.08	0.2±0.07	0.18±0.04*	0.37±0.05	0.31±0.03	0.34±0.02*

Table 6. Organ to body weight ratios of transgenic mice.

The organ to body weight ratios of male and female *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice (aged 10-14 weeks) are given in the table above. Data are provided as mean ± standard deviation (n=3). Differences in ratios between female and male mice are highlighted (*) when $P < 0.05$.

3.5.2 PrdxVI

Peroxiredoxins are a family of selenium-independent antioxidants involved in the reduction of hydroperoxides to alcohols (Wood et al., 2003). There are two main families of peroxiredoxins; those containing two conserved cysteine residues in their C-terminal domain (2-Cys) and those only containing one cysteine residue (1-Cys). The lack of an extra cysteine molecule for the 1-Cys family, or peroxiredoxin VI (PrdxVI), means that another molecule containing a thiol group is needed in order to reduce the oxidised Cys 47 molecule and to regenerate the active PrdxVI (Choi et al., 1998). It has been suggested that GSTP1 provides the thiol group required in a glutathionylation step by forming a heterodimer with PrdxVI (Manevich et al., 2004) and it has been demonstrated in vitro that a stable GSTP1:Cys1 complex could be formed which restores active PrdxVI activity (Ralat et al., 2006). This example of GSTP1 mediated glutathionylation of cellular proteins is discussed further in Chapter 4. However, further data suggest interplay in the regulation of these two proteins, in that deletion of GSTP1 results in the upregulation of PrdxVI protein expression in mice bred on a C57x129 background (Kitteringham et al., 2003). In our studies, we could not detect any difference in PrdxVI expression in the livers of *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice generated on a C57Bl/6J background (Figure 3.5). This result may allude to differences in mouse strain, and perhaps is a result of different function of Ala^{124} and Asp^{124} polymorphic variants of PrdxVI, observed in C57Bl/6J and 129 mouse strains, respectively.

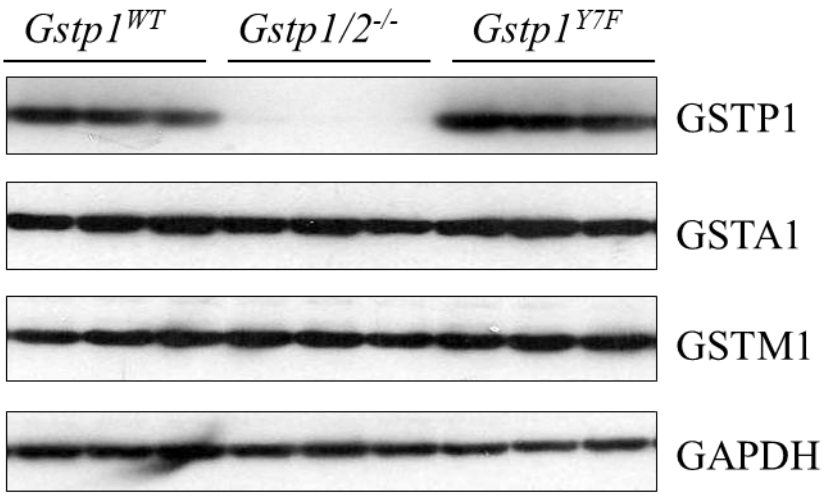


Figure 3.4. Protein expression profile of *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice.

Hepatic cytosolic fractions (10µg) from male mice (n=3) were resolved on a SDS PAGE gel and proteins analysed by Western blot analysis. Blots demonstrate the presence of GSTP1 in *Gstp1*^{WT} and *Gstp1*^{Y7F} mice and its absence in *Gstp1/2*$^{-/-}$ mice. The blots demonstrate that there is no apparent compensation in the expression of other GSTs.

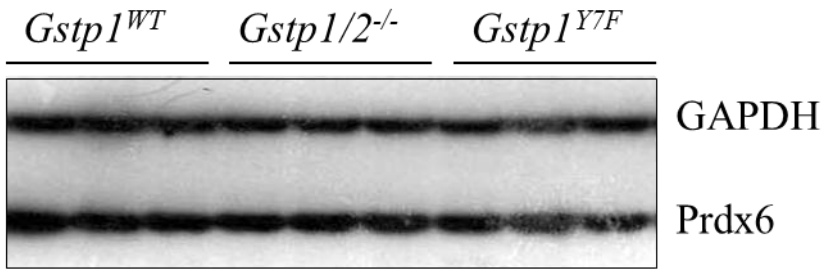


Figure 3.5. PrdxVI expression in the livers of *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice.

Hepatic cytosolic fractions (10µg) from male mice (n=3) were resolved on a SDS PAGE gel and proteins analysed by Western blot analysis. Blots demonstrate that there is no change in the expression of PrdxVI protein levels in either *Gstp1/2*$^{-/-}$ or *Gstp1*^{Y7F} mice.

3.5.3 Localisation

Although widely distributed in a range of tissues, in mice GSTP1 is expressed largely in the liver and can be found in hepatocytes but not in Kupffer cells, bile duct cells and endothelial cells (Vaughn et al., 2011). Using an anti-GSTP1 antibody, immunohistochemical analysis shows that the expression of GSTP1 in wild-type mice appears to localise in hepatocytes predominantly within midzonal regions of the liver, although expression is observed in centrilobular regions (Figure 3.6). GSTP1 expression in *Gstp1*^{Y7F} mice localises to similar regions and cells within the liver, which suggests that not only is the non-catalytic protein expressed to the same extent as its wild-type counterpart, but that it is also localised to the same cellular compartments. Similarly to that observed in Figure 3.4, no GSTP1 protein can be detected in *Gstp1/2*$^{-/-}$ liver sections, highlighting the specificity of the antibody for GSTP1.

3.5.4 Catalytic activity of GSTP1 in mouse liver

The catalytic activity of GSTP1 in mouse liver was determined spectrophotometrically using ethacrynic acid. Due to its cross reactivity with other GSTs, CDNB is unsuitable as a substrate due to the presence of GSTM and GSTA classes within cytosolic fractions (Figure 3.4). Although it has been shown to cross react with other GST classes (Ploemen et al., 1993), ethacrynic acid is a potent inhibitor of GSTP1 and is commonly used to determine the enzymatic activity of GSTP1. Figure 3.7 shows that only hepatic cytosolic fractions prepared from *Gstp1*^{WT} mice exhibits activity towards ethacrynic acid, demonstrating that GSTP1 present in *Gstp1*^{Y7F} mouse liver is catalytically redundant.

Incorporating both the protein expression and catalytic data, we can confidently assume that *Gstp1*^{Y7F} mice express a GSTP1 protein which is expressed and localised to the same extent

in the liver as its wild-type counterparts but is catalytically inactive. Therefore, any subsequent phenotype observed in these mice is a result of the catalytic function of GSTP1. However, we must acknowledge that these mice still retain the *Gstp2* gene and, although transcriptionally and catalytically redundant, may have a non-catalytic role in cytoprotection. However, as both $Gstp1^{WT}$ and $Gstp1^{Y7F}$ mice carry the *Gstp2* gene, it is unlikely to account for any difference in phenotype. It is also important to acknowledge that there may be some residual catalytic activity as a result of the Y7F mutation that has not been detected by the ethacrynic acid assay but is clearly evident from recombinant protein studies (Table 5).

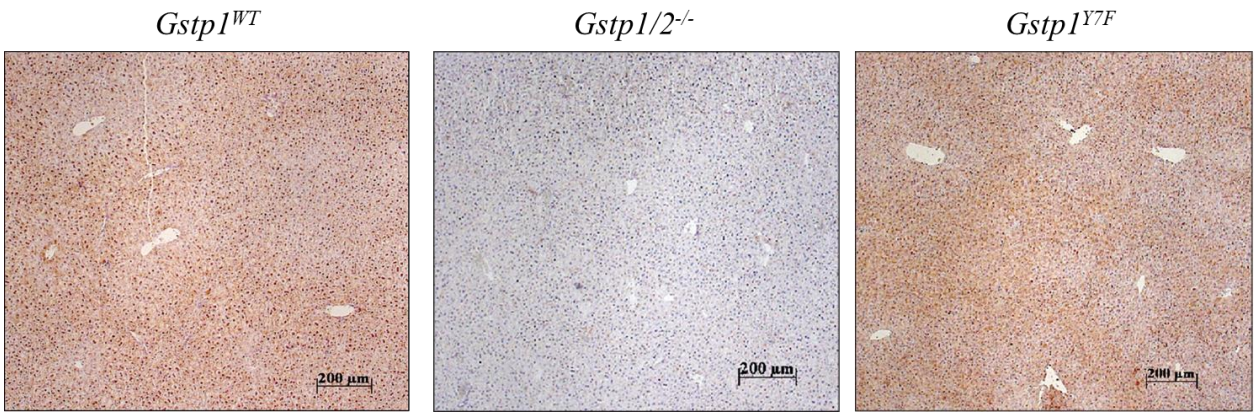


Figure 3.6. Immunohistochemical staining of GSTP1 in mouse liver

Localisation of GSTP1 was observed in formalin fixed tissue sections (5µm) from *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} male mouse livers using an anti-GSTP1 antibody (1:500). Sections are representative of 3 separate animals.

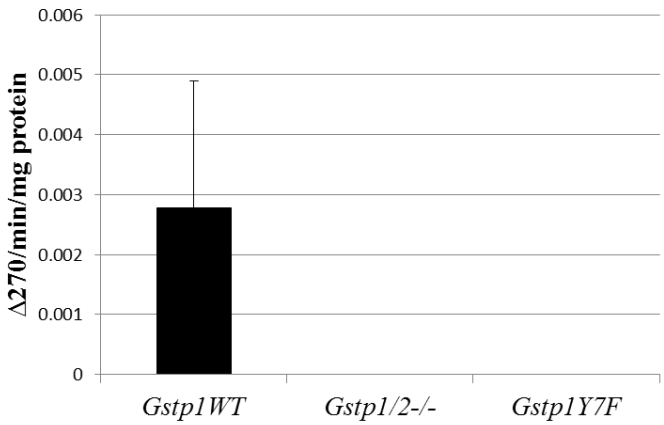


Figure 3.7. Assessing catalytic activity of GSTP1 in cytosolic mouse liver fractions.

The enzymatic activity of hepatic cytosolic fractions prepared from *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} male mouse livers (=3) towards ethacrynic acid was measured spectrophotometrically at 270nm.

3.6 Acetaminophen (APAP) treatment of *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice

Initially described on a 129xMF1 background, the ability of *Gstp1/2*$^{-/-}$ mice to regulate their hepatic glutathione levels in response to acetaminophen treatment is a potential mechanism in which toxicity is mitigated, as pharmacokinetic studies show no or little difference in the way acetaminophen is metabolised between *Gstp1*^{WT} and *Gstp1/2*$^{-/-}$ mice (Henderson et al., 2000). The use of acetaminophen in this study was not to establish translational effects of acetaminophen hepatotoxicity in humans, as human liver contains little GSTP1, but to establish the function of mGSTP1 in response to hepatic stress using a well characterised model of hepatotoxicity. With the development of a catalytically inactive GSTP1 mouse model, we can now address whether resistance is only acquired in the absence of GSTP1, or whether the catalytic activity of GSTP1 is important in contributing to acetaminophen toxicity. Male *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice, aged between 8-16 weeks old were administered a single dose of acetaminophen (300mg/kg body weight, oral gavage) and left for 24 hours. Mice were starved for 16 hours prior to acetaminophen treatment to ensure complete absorption of acetaminophen across the gastrointestinal tract. Mice were age matched across groups. Alanine aminotransferase (ALT) is present within liver hepatocytes and catalyses the amino transfer from alanine to α-ketoglutarate. When the liver is damaged, ALT is leaked into the blood and can be used to assess the extent of hepatic damage after exposure to a given toxicant. Lactate dehydrogenase (LDH) release into the bloodstream can also be used as a measure of tissue damage, although results are less specific of liver damage than ALT. After 24 hours, mice were sacrificed and plasma ALT and LDH levels were determined to assess hepatotoxicity (Figure 3.8A and Figure 3.8B respectively). After 24 hours, plasma ALT and LDH levels were significantly higher in acetaminophen treated wild-type mice compared to saline (control) treated mice, indicating a large degree of hepatic damage as a result of acetaminophen treatment. Plasma bilirubin and creatinine levels

remained unchanged (data not shown). No increase in ALT or LDH plasma concentrations were detected in acetaminophen treated *Gstp1/2*$^{-/-}$ mice which correlates with previous studies showing that *Gstp1/2*$^{-/-}$ mice are resistant to the hepatotoxic effects of acetaminophen (Henderson et al., 2000, Vaughn et al., 2011). Similar to *Gstp1/2*$^{-/-}$ mice, *Gstp1*^{Y7F} mice show no increase in ALT or LDH plasma concentrations 24 hours after acetaminophen treatment, suggesting that these mice are also resistant to the hepatotoxic effects of acetaminophen.

The majority of publications involving *Gstp1/2*$^{-/-}$ mice have been using mice bred from an outbred (129xMF1) background. It is well documented that differences in mouse strain may account for differences in susceptibility to a given toxicant or mutational status, with some inbred strains showing differences in response to acetaminophen treatment (Harrill et al., 2009b, Turk et al., 2004, Silva et al., 1997). Not only are the data presented here agreeable with previous publications on *Gstp1/2*$^{-/-}$ resistance to acetaminophen, but they also show a common phenotype of *Gstp1/2*$^{-/-}$ mice across two different mouse strains, 129xMF1 and C57Bl/6J. This suggests that original observations made by Henderson et al, were not influenced by mouse strain selection and that the catalytic function of GSTP1 appears to be the main factor behind acetaminophen toxicity in this study.

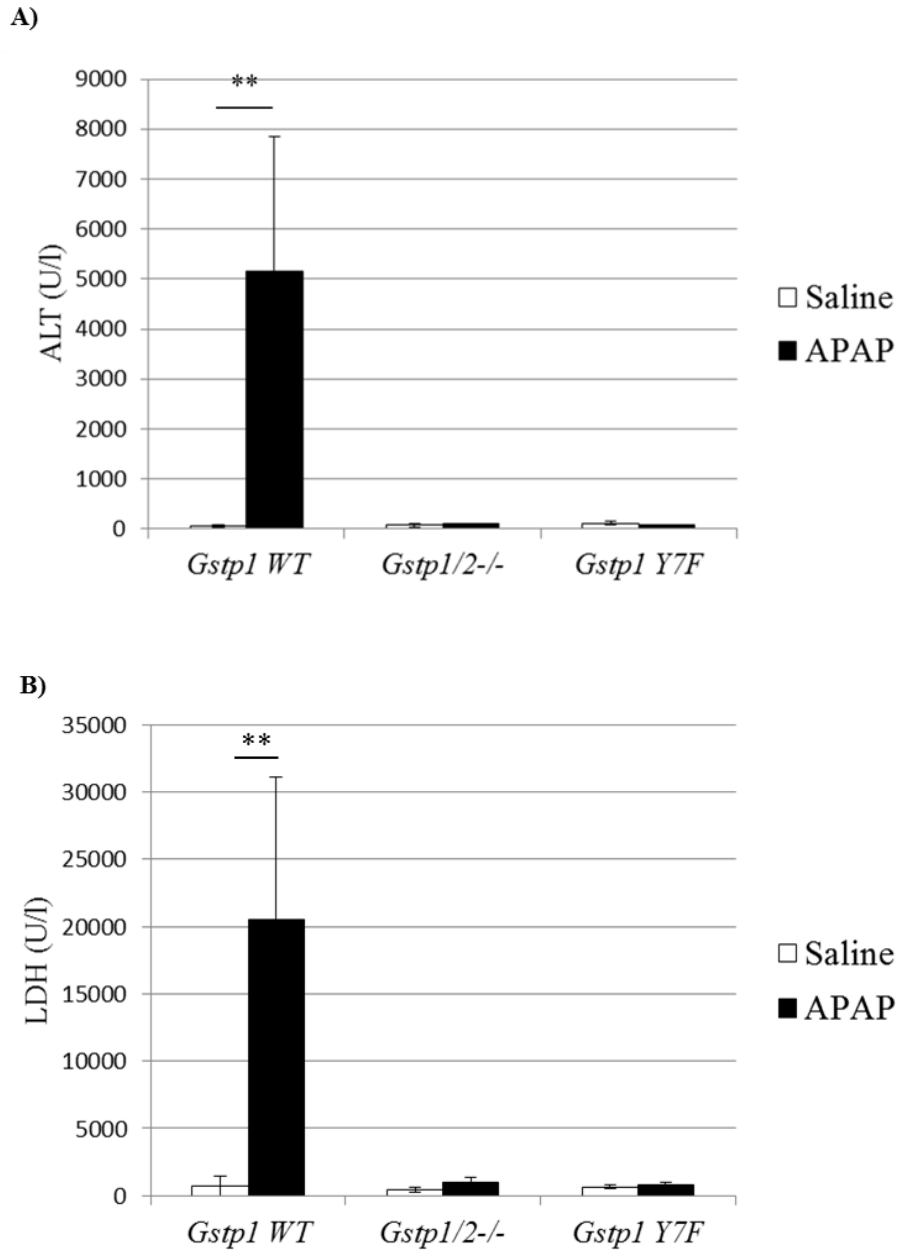


Figure 3.8. Plasma ALT and LDH levels in mice treated with acetaminophen.

Male *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice were administered a single dose of saline or acetaminophen (300mg/kg, oral gavage, n=3) and sacrificed after 24 hours. The blood was removed, heparinised and ALT and LDH levels were determined. Data show mean ± standard deviation values for ATL (**A**) and LDH (**B**). ** Statistical difference between saline and acetaminophen treated mice, $P < 0.01$.

The extent of liver damage in acetaminophen treated mice was determined by histochemical examination of liver tissue (Figure 3.9). Immunohistochemical staining of mouse liver sections following acetaminophen treatment shows extensive centrilobular necrosis as a result of acetaminophen treatment in *Gstp1*^{WT} mice (Figure 3.9) which correlates with increased plasma ALT and LDH levels. The level of oxidative stress was also assessed from these sections. Haem oxygenase-1 (HO-1) catalyses the cleavage of haem into iron, carbon monoxide and biliverdin and can be induced to a diverse set of stimuli including UV radiation, oxidative stress nitrosative stress, ethanol, inflammation and heavy metals. Activity of HO-1 reduces the levels of oxidative stress in the cell, primarily through the removal of haem, but also through the activity and properties of the by-products of haem degradation. In our studies, 24 hours after acetaminophen treatment showed that surrounding regions of hepatic necrosis exhibited increased expression of HO-1, indicative of increased stress response to acetaminophen treatment. No liver damage could be detected in *Gstp1/2*$^{-/-}$ or *Gstp1*^{Y7F} mice after 24 hours of a single dose of acetaminophen. However, HO-1 expression was evident around the centrilobular regions of acetaminophen treated livers in these mice, suggesting that although resistant to the hepatotoxicity of acetaminophen, a stress response is still apparent in the livers of these mice. This indicates that in the absence of a catalytically functioning GSTP1, acetaminophen-induced oxidative stress is nullified to a greater extent resulting in attenuation of hepatotoxicity.

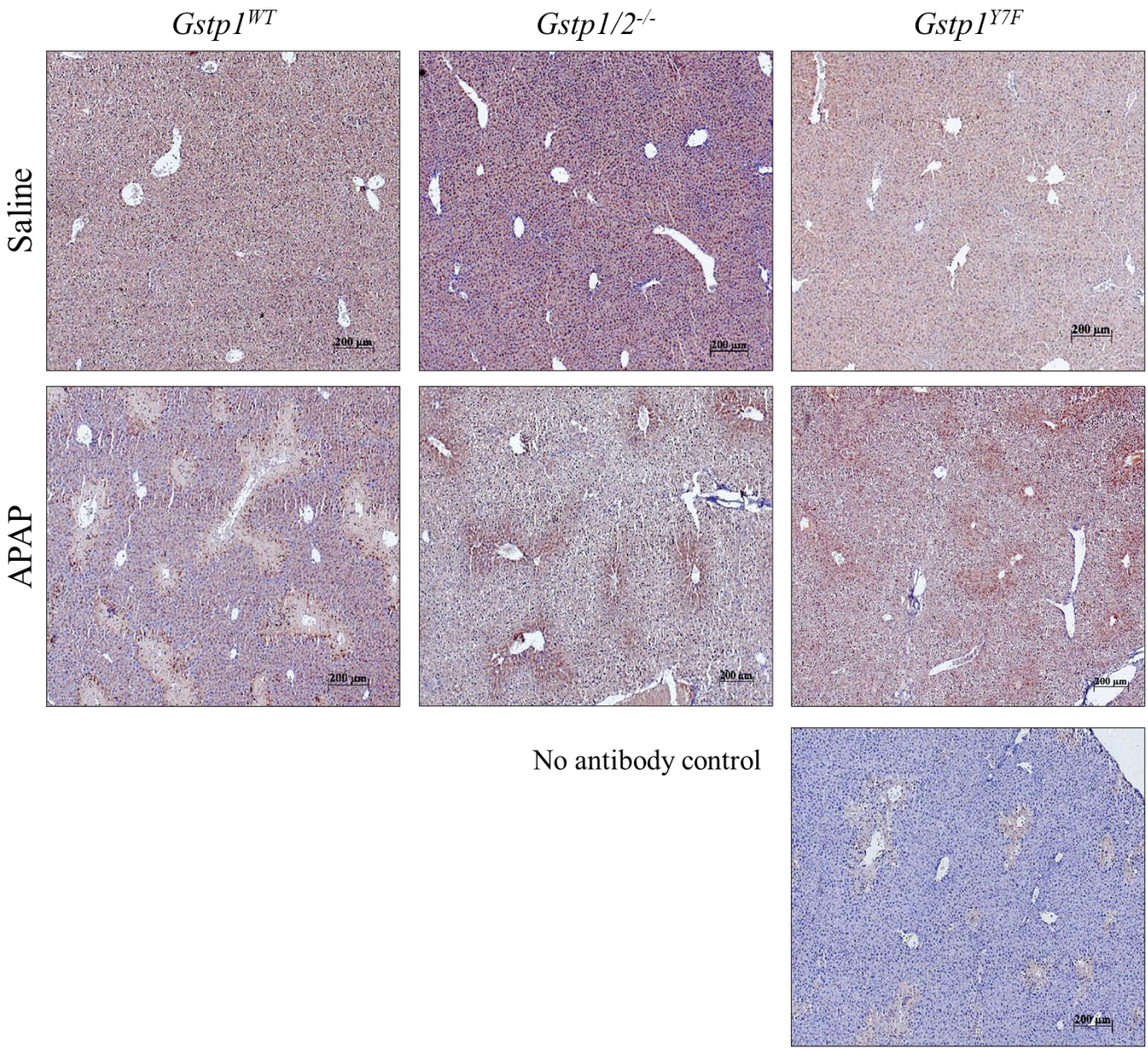


Figure 3.9. Liver immunohistochemistry of haem oxygenase-1 in acetaminophen treated mice.

Male *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice were administered a single dose of saline or acetaminophen (300mg/kg, oral gavage) and sacrificed after 24 hours. Livers were removed, fixed and cut into 5µm sections before stained for haem oxygenase-1 expression (HO-1, 1:100). Sections are representative of 3 separate animals.

3.7 Hepatic glutathione levels following acetaminophen treatment

A possible mechanism explaining the resistance of *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice against acetaminophen toxicity could be in the regeneration of hepatic glutathione levels, as glutathione is rapidly depleted from the liver following acetaminophen treatment. Male *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice aged 10-16 weeks were administered a single dose of acetaminophen (300mg/kg, oral gavage) after an initial 16hr starvation period. The mice were then sacrificed at 20, 40, 90 and 240 minutes and the liver and blood removed. To ensure accuracy of analysis across all samples taken, lobe 2 of the liver was used for glutathione analysis. Resting levels of both total and disulphide glutathione (GSSG) did not vary greatly between genotypes as shown in Figure 3.10A and Figure 3.10B respectively. Rapid depletion of glutathione was evident upon acetaminophen treatment, with maximum hepatic glutathione depletion occurring at 90 minutes for all genotypes (Figure 3.10A). However, the rate of glutathione depletion was more dramatic in *Gstp1*^{WT} livers where maximum depletion occurred after 40 minutes. In *Gstp1*^{WT} mice hepatic glutathione levels remained depleted by 240 minutes, whereas hepatic glutathione was fully regenerated by this time point in *Gstp1/2*$^{-/-}$ mice. *Gstp1*^{Y7F} mice only achieved partial recovery of hepatic glutathione levels (41.6%) by 240 minutes. The ability of *Gstp1*^{Y7F} mice to only partially regenerate hepatic glutathione levels appears sufficient to avoid the hepatotoxic effects of acetaminophen and is in line with other studies showing that covalent binding of proteins adducts and, as a result of which, subsequent necrosis only occurs at maximal (80-90%) glutathione depletion in hepatocytes (Jollow et al., 1974, Green et al., 1984). However, hepatic glutathione concentrations reached similar levels of depletion in each genotype suggesting that a mechanism downstream of glutathione depletion is responsible for the toxicity and not the initial depletion itself.

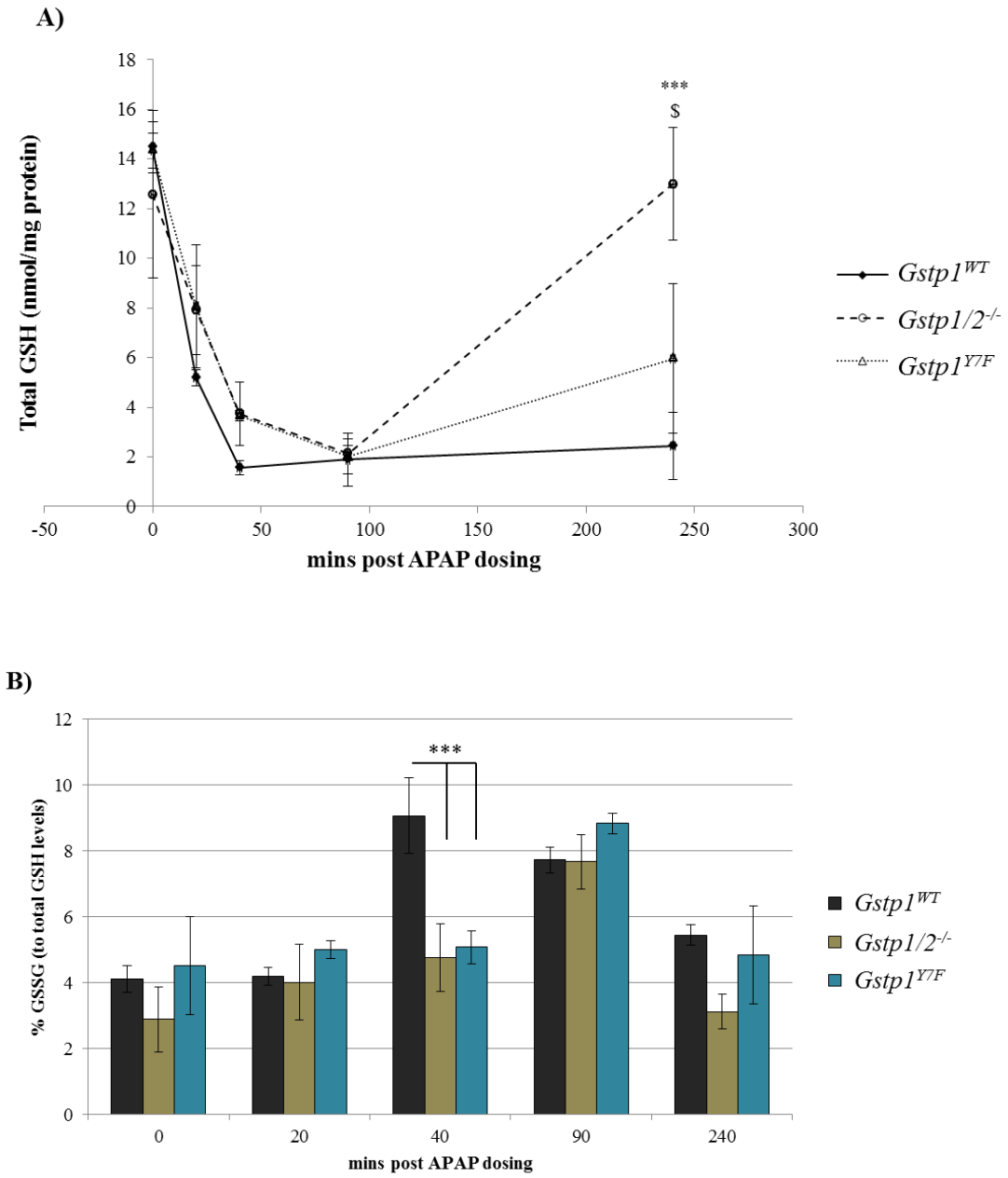


Figure 3.10. Hepatic glutathione levels in mice following acetaminophen treatment

Male *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice were administered a single dose of saline or acetaminophen (300mg/kg, oral gavage, n=3) and sacrificed at the time points shown. Livers were removed and analysed for **A)** total levels of glutathione and **B)** disulphide levels of glutathione. Data are presented as mean ± standard deviation. Statistical difference is shown between wild-type mice and *Gstp1/2*$^{-/-}$/*Gstp1*^{Y7F}mice where *** P <0.001. Statistical difference between *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F}mice is shown where $, P <0.01

In this study, maximal depletion of glutathione in *Gstp1*^{WT} mice occurs after 40 minutes of acetaminophen treatment and corresponds with a significant increase in the proportion of GSSG (Figure 3.10B). As maximal depletion of glutathione occurs after 90 minutes in *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice, we observe a delayed response in the oxidation of glutathione in absence of a catalytically functioning GSTP1. However, at this time point, there is no difference in levels of GSSG across the genotypes. It is also interesting to note that despite the differences in total glutathione after 240 minutes, there is no statistical difference in the proportion of GSSG across the genotypes, suggesting that oxidative stress is not a mechanism by which hepatotoxicity is potentiated in *Gstp1*^{WT} mice. This observation is strengthened further when examining HO-1 expression from liver samples taken from *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice (Figure 3.11). Western blot analysis of whole cell lysates demonstrates increased HO-1 expression after 240 minutes across all genotypes, implying that although oxidative stress response is evident in the livers of these mice, it is not indicative of the severity of the toxicity. This correlates well with our previous observations examining HO-1 localisation in mouse liver 24 hours post acetaminophen treatment (Figure 3.9).

3.8 Activation of MAP Kinase signalling

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine-specific protein kinases that, in response to a diverse set of stimuli, regulate a number of pathological pathways, such as differentiation, inflammation, stress response and apoptosis, through a series of phosphorylation cascades. The MAPKs are composed of 3 major subfamilies, the extracellular signal regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and the p38 MAPKs. Activation of MAPKs occurs through a three tiered cascade whereby a given stimulus activates a MAPK kinase kinase (MKKK/MEKK), which in turn phosphorylates a MAPK kinase (MAPKK) upstream of MAPK. MAPKs have been shown to have overlapping

substrate specificities as well as demonstrating interactions between signalling cascades (Staples et al., 2010). Where ERK activation is predominantly regulated by hormones and growth factors, the JNK and p38 family of MAPKs are activated in response to a variety of stressors such as inflammation, environmental stress, oxidative stress and DNA damage. Regulation of these pathways is mediated through MAPK phosphatases (MKPs) which negatively regulate the activation of these cascades through dephosphorylation of MAPKs (Owens and Keyse, 2007), although there is evidence to suggest that ubiquitylation of upstream MEKKs may feature in the negative regulation of some MAPKs (Witowsky and Johnson, 2003).

Regulation of JNK and ERK pathways has been shown to be an important factor in hepatocellular processes such as hepatocyte apoptosis and steatohepatitis induced by non-alcoholic fatty liver disease (NAFLD) (Qiao et al., 2003, Schattenberg et al., 2006). In response to stress, JNK phosphorylation results in the activation and stabilisation of a number of its transcription targets such as c-Jun, JunB, p53 and ATF-2 which in turn activate a number of cellular processes. The activation of JNK in response to acetaminophen treatment has been demonstrated as a potential mechanism by which toxicity may be propagated. Blocking JNK phosphorylation using a small molecule inhibitor protects mice against acetaminophen induced hepatotoxicity (Gunawan et al., 2006), while a lack of JNK phosphorylation in *SOD1*$^{-/-}$ mice has been attributed to a decrease in sensitivity to acetaminophen (Zhu et al., 2006). In contrast, the use of JNK knockout mice in determining the role of JNK in response to acetaminophen has been contradictory. The JNK family is encoded by 3 distinct genes, *Jnk1, Jnk2* and *Jnk3*. *Jnk1* and *Jnk2* are ubiquitously expressed, whereas *Jnk3* is mainly localised to the nervous system. Deletion of both *Jnk1* and *Jnk2* is embryonically lethal. Deletion of individual isoforms of JNK has demonstrated inconsistent

changes in sensitivity to acetaminophen, with reports differing on the sensitivity of these mice, or demonstrating no sensitivity whatsoever (Gunawan et al., 2006, Bourdi et al., 2008). However, a recent study by Wancket et al may shed light on the mechanism by which JNK activation causes hepatotoxicity. In mice nulled for *Mkp-1*, which negatively regulates JNK through dephosphorylation, acetaminophen treatment induces gross hepatotoxicity compared to wild-type mice (Wancket et al., 2012), suggesting that the regulation of JNK phosphorylation, and not necessarily its activation, is important in the propagation of hepatotoxicity.

In response to a single dose of acetaminophen (300mg/kg, oral gavage), Western blot analysis demonstrates increased phosphorylation of both JNK1 and JNK2 isoforms in *Gstp1*^{WT} mice at 90 and 240 minutes post acetaminophen dosing (Figure 3.11). Phosphorylation of MAP kinase pathways does not appear exclusive to JNK1/2 as ERK1/2 activation is also apparent in these mice. Interestingly, phosphorylation of ERK1/2 appears to occur earlier than phosphorylation of JNK1/2. In *Gstp1*^{WT} mice, kinase phosphorylation is sustained for up to 240 minutes when glutathione depletion is maximal. In contrast, *Gstp1/2*$^{-/-}$ mice have increased JNK1/2 and ERK1/2 phosphorylation at 90 minutes, but MAPK phosphorylation is lost at 240 minutes when a full recovery of hepatic glutathione levels is observed. *Gstp1*^{Y7F} mice display a similar profile to that of their wild-type counterparts, showing sustained JNK1/2 phosphorylation at 90 and 240 minutes post acetaminophen dosing. Although ERK1/2 phosphorylation is also evident, it appears markedly reduced compared to *Gstp1*^{WT} mice, correlating well with a partial recovery of hepatic glutathione levels. Phosphorylation of ERK1/2 is also evident from immunohistochemical analysis of liver sections from these mice (Figure 3.12).

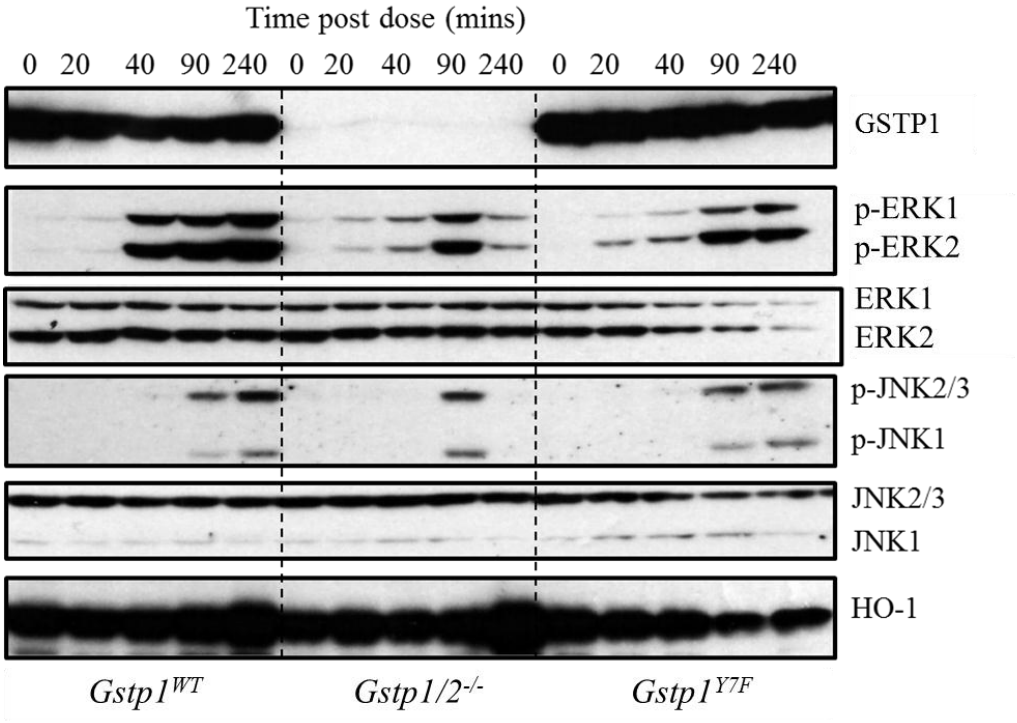


Figure 3.11. Expression of markers of MAP Kinase signalling in response to acetaminophen treatment.

Male *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice were administered a single dose of saline or acetaminophen (300mg/kg, oral gavage, n=3) and sacrificed at the time points shown. Livers were removed and whole cell lysates were prepared. Pooled lysates from 3 animals (10µg) were resolved on a SDS PAGE gel and proteins analysed by Western blotting.

Figure 3.12 demonstrates phosphorylation of ERK1/2 around centrilobular regions of the liver in *Gstp1*^{WT} and *Gstp1*^{Y7F} mice in response to acetaminophen, but is largely attenuated in *Gstp1/2*$^{-/-}$ mice. These data demonstrate a positive correlation between activation of MAP kinase pathways and hepatic glutathione levels, suggesting that acetaminophen-induced hepatotoxicity is associated with the activation of MAP kinase activation as a result of glutathione depletion. These studies show a potential mechanism by which *Gstp1/2*$^{-/-}$ mice

appear resistant to the toxicity of acetaminophen. However, due to the resistance of *Gstp1*^{Y7F} mice to acetaminophen, it is unlikely that the lack of MAPK activity in *Gstp1/2*$^{-/-}$ mice is solely responsible for resistance against acetaminophen.

3.9 Mitochondrial localisation and function of GSTP1

It is well documented that mitochondrial damage as a result of acetaminophen treatment can contribute to hepatocellular necrosis. In particular, changes to mitochondrial membrane permeability in response to a number of stressors, such as calcium ion increase and oxidative stress can lead to depolarisation of the mitochondrial membrane, uncoupling of respiratory chains and mitochondrial leakage (Ray et al., 1993, Burcham and Harman, 1991, Weis et al., 1992). Blockage of the mitochondrial permeability transition pore (MPTP) by Cyclosporine A and its analogues leads to a reduction in acetaminophen induced toxicity in hepatocytes (Kon et al., 2004). Increased levels of peroxide formation in mitochondria have been reported in response to acetaminophen treatment (Cover et al., 2005) while recent evidence suggests that the activity of manganese superoxide dismutase (MnSOD) localised within the mitochondria is significantly reduced due to nitrosylation of the enzyme in the presence of NAPQI (Agarwal et al., 2011).

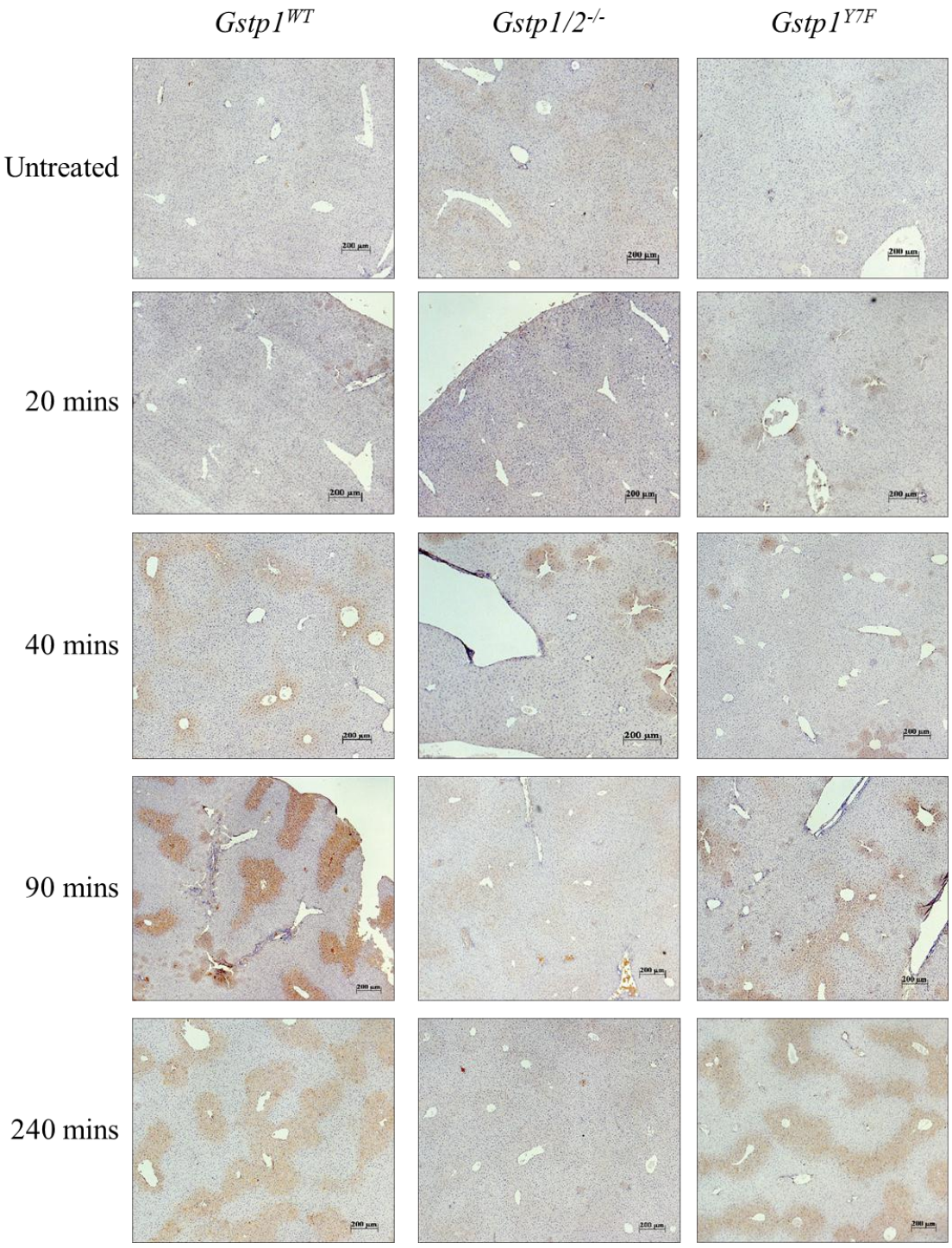


Figure 3.12. Liver immunohistochemistry of phosphorylated ERK in acetaminophen treated mice.

Male *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice were administered a single dose of saline or acetaminophen (300mg/kg, oral gavage) and sacrificed at the time points shown (n=3). Livers were removed, fixed and cut into 5µm sections before stained for p-ERK expression (1:100). Sections are representative of 3 separate animals.

There are a number of recent studies which suggests that GSTP1 is present within mammalian mitochondria although its function is still largely unknown (Sun et al., 2012, Gallagher et al., 2006). Goto et al demonstrated that GSTP1 is present in the mitochondria of a number of cancer cell lines and, through site-directed mutagenesis, determined that its localisation is dependent on a series of positively charged residues in its N-terminal region (Goto et al., 2009). In our studies, we are interested in whether mGSTP1 localisation and function within the mitochondria can lead to differences in acetaminophen sensitivity. Using immunogold labelling of a GSTP1 antibody, we aimed to identify whether GSTP1 can localise to the mitochondria in mouse liver, and if its expression changes in response to acetaminophen treatment. In line with previous reports, at resting levels we can identify GSTP1 within hepatocytes and determine that its expression within the mitochondria is much lower compared to its expression in the cytoplasm (Figure 3.13A). We also observe that the Y7F mutation does not affect the localisation of GSTP1 within the mitochondria (Figure 3.13A). In response to a single oral dose of acetaminophen (300mg/kg), GSTP1 does not appear to increase its mitochondrial or cytoplasmic expression in *Gstp1*^{WT} mice (Figure 3.13B). The expression of GSTP1 in these mice appears reduced 24 hours after acetaminophen treatment; however the apparent hepatic necrosis associated with acetaminophen treatment at this time point may account for this observation. GSTP1 expression is evident in *Gstp1*^{Y7F} mice prior to and after acetaminophen treatment although no apparent change in subcellular localisation is evident after acetaminophen treatment. As negative controls, no GSTP1 protein could be detected in *Gstp1/2*$^{-/-}$ mice or in the absence of the primary antibody.

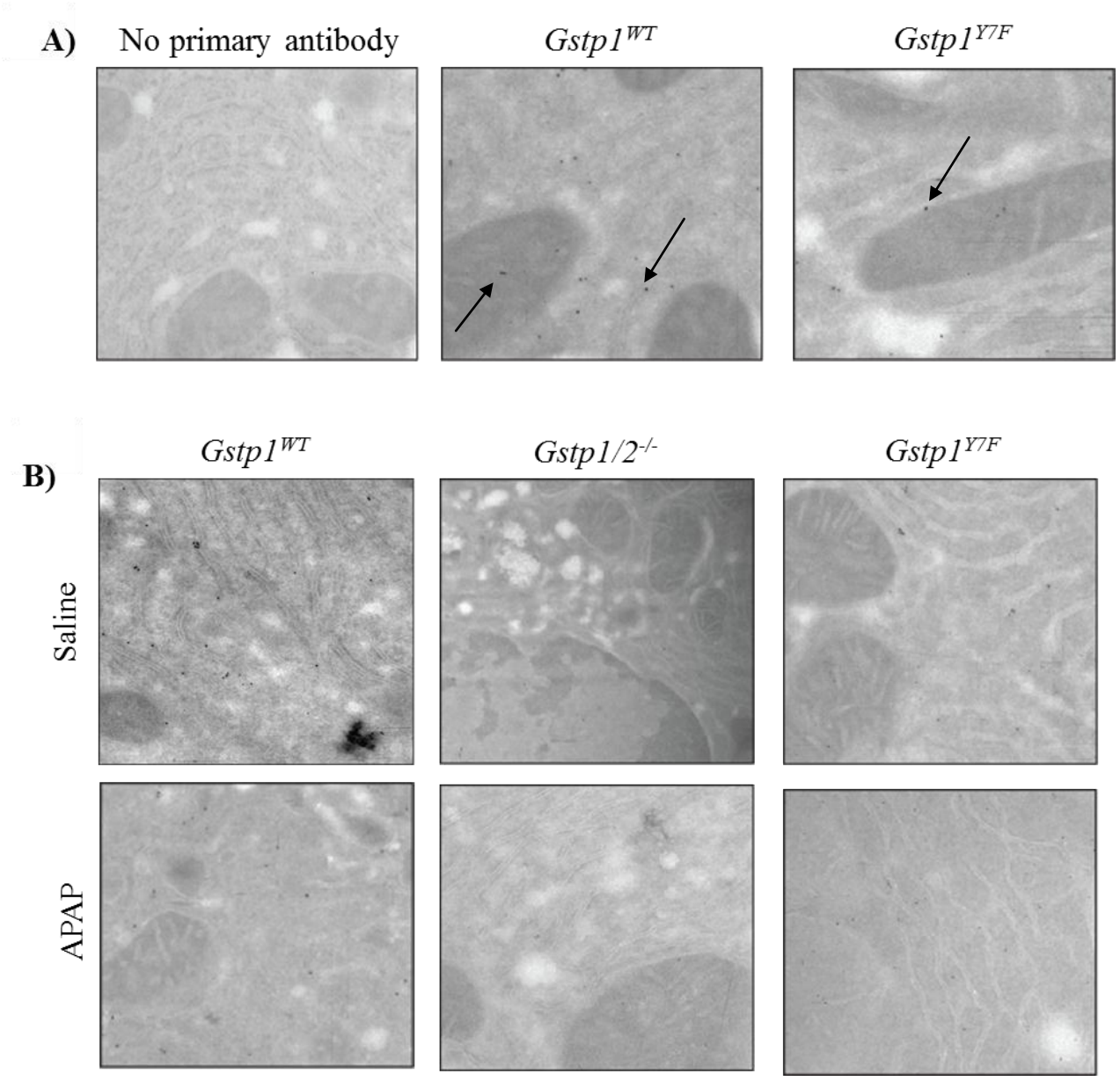


Figure 3.13. Mitochondrial localisation of GSTP1 in response to acetaminophen treatment.

Male *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice were administered a single dose of saline or acetaminophen (300mg/kg, oral gavage) and sacrificed after 24 hours (n=1). Livers were removed and fixed before intracellular localisation of GSTP1 was evaluated using immunogold labelling of a GSTP1 antibody (black arrows) and analysed by electron microscopy (B). The basal expression of GSTP1 within the mitochondria was also evaluated (A). All images are at a magnification of 25,000x.

To assess whether GSTP1 has an active function within the mitochondria, mouse embryonic fibroblasts (MEFs) were isolated from *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice and incubated with rotenone, which inhibits complex I of the mitochondrial respiratory chain and thereby inhibiting mitochondrial respiration. Paradoxically, after 72 hours of rotenone treatment, there is a slight increase in resistance to rotenone in *Gstp1/2*$^{-/-}$ MEFs compared with *Gstp1*^{WT} or *Gstp1*^{Y7F} MEFs (Figure 3.14). Although not statistically significant, the results may indicate a potential and novel function of GSTP1 within the mitochondria in response to stress.

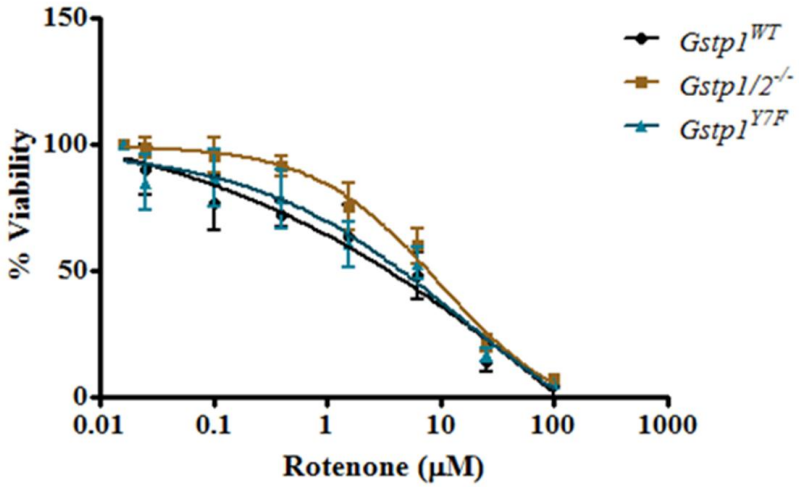


Figure 3.14. Cytotoxic effect of rotenone in *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} MEFs.

Mouse embryonic fibroblasts (MEFs) isolated from *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice were incubated with rotenone over 72 hours (n=3). The data show mean ± standard deviation.

To further examine the role of GSTP1 within the mitochondria, levels of oxygen consumption and glycolysis were determined in response to mitochondrial stress using the XF24 Analyser (Seahorse Biosciences). Cells were plated at 20,000 cells per well and basal

oxygen consumption and glycolytic rates were measured before incubating cells with 100µM of 2,4-dinitrophenol (DNP), which uncouples respiration from ATP synthesis and thereby increasing both mitochondrial oxygen consumption and glycolysis. In 2007, Wu et al demonstrated the specificity of the XF Analyser in measuring glycolytic and respiratory rates using a number of compounds which act differentially on mitochondrial function (Wu et al., 2007). At resting levels, we observe marked differences in oxygen consumption between genotypes, with *Gstp1/2*$^{-/-}$ MEFs possessing a much lower oxygen consumption rate (OCR) than *Gstp1*^{WT} or *Gstp1*^{Y7F} MEFs (Figure 3.15A). In response to DNP treatment, there is a significant increase in OCR across all genotypes observing 2.6 and 3.2 fold increases in OCR in *Gstp1*^{WT} and *Gstp1*^{Y7F} MEFs, respectively. Despite a lower rate of OCR in response to DNP the fold difference increases dramatically to 4.9 in *Gstp1/2*$^{-/-}$ MEFs indicating that, while basal oxygen consumption is lower, mitochondria lacking GSTP1 are respiring at a larger rate in response to stress. This may in part explain why the effects of rotenone, which inhibits mitochondrial respiration, are attenuated in *Gstp1/2*$^{-/-}$ MEFs.

It is interesting to note that there is no difference in basal glycolytic rates (ECAR) across genotypes (Figure 3.15B). When exposed to DNP however, ECAR dramatically increases in *Gstp1*^{WT} and *Gstp1*^{Y7F} MEFs due to increased lactic acid formation, yet only mildly increases in *Gstp1/2*$^{-/-}$ MEFs. Firstly, this demonstrates that the presence of GSTP1 and not its catalytic function is responsible for this increase, highlighting potential non-catalytic functions of GSTP1 within the mitochondria. Secondly, the observation that glycolytic rate in *Gstp1/2*$^{-/-}$ MEFs was much lower than other genotypes, while the fold difference in OCR was much higher suggests that perhaps mitochondria favour oxidative phosphorylation over glycolysis in response to stress in the absence of GSTP1.

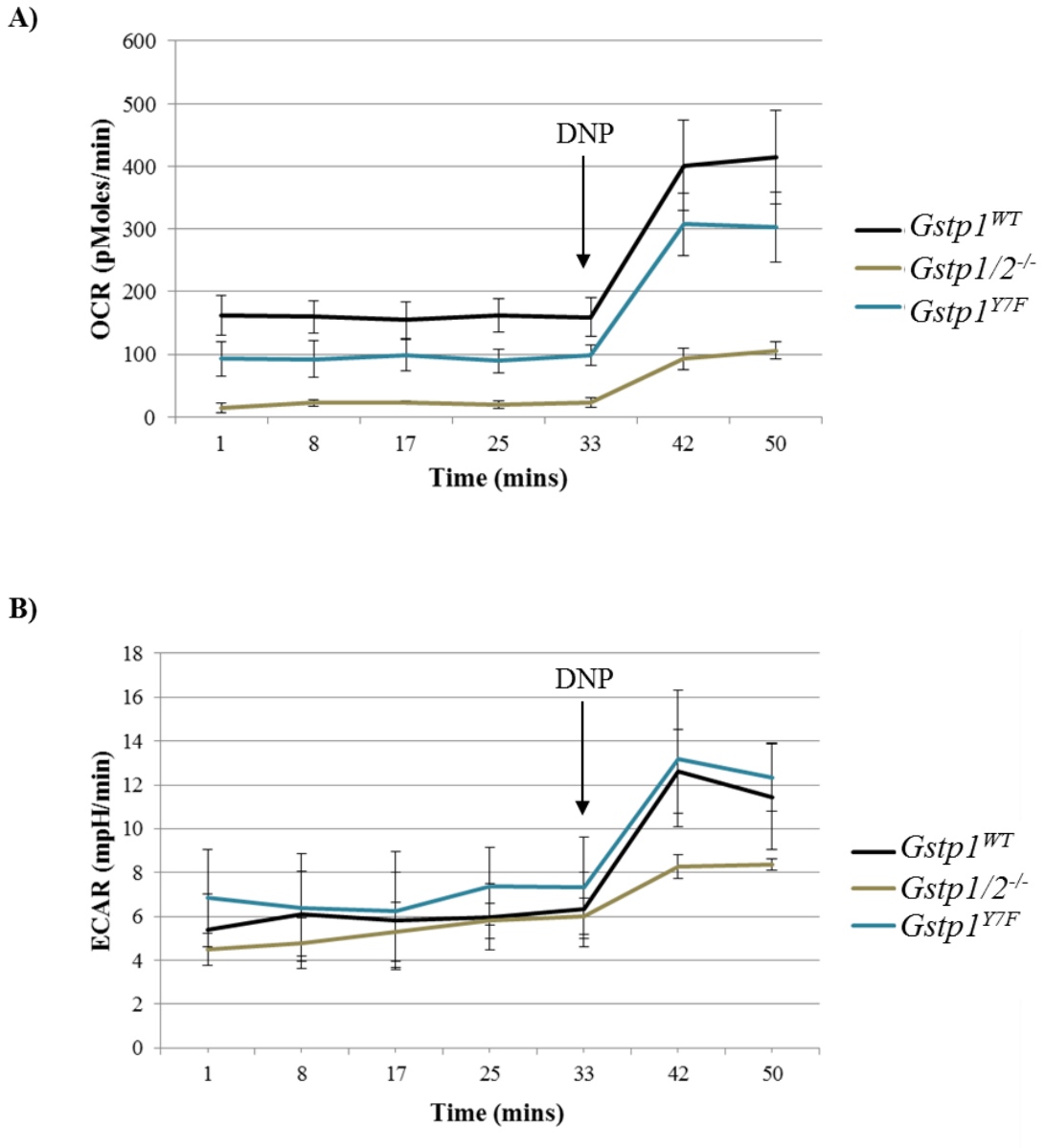


Figure 3.15. Mitochondrial respiratory function of GSTP1 in response to 2,4-dintrophenol (DNP).

Oxygen consumption rates **(A)** and levels of glycolysis **(B)** were analysed in mouse embryonic fibroblasts (MEFs) isolated from *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice using the XF24 Analyser (n=3). Cells were incubated in unbuffered media for 1 hour prior to analysis. After 5 initial readings, cells were treated with DNP (100µM). Data show mean ± standard deviation.

In summary, a Y7F substitution results in a GSTP1 protein which is catalytically inactive *in vivo*. Initial studies suggest that the catalytic function of GSTP1 is not essential for murine development but contributes to the hepatotoxicity associated with APAP. *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice do not develop hepatic necrosis after APAP treatment and are able, in part, to regenerate hepatic GSH levels in response to APAP. This phenotype does not appear to be related to oxidative stress but relies on the activation of particular MAP Kinase pathways. Further studies are required to determine if the mitochondrial function of GSTP1 may play a role in propagating this effect, although preliminary data suggest that GSTP1 may act non-catalytically in mediating mitochondrial regulation.

4. Chapter 4: GSTP1-mediated protein S-glutathionylation of cellular proteins

Introduction

Chapter 3 largely examined the *in vivo* function of GSTP1 in relation to a hepatotoxic compound; the exact mechanism by which GSTP1 contributes to this phenotype is still not fully understood. Specific GSTP1-mediated cell regulation has been difficult to ascertain *in vivo* due to the complexity of stress response pathways and upregulation of compensation mechanisms as a result of loss of function. Therefore examining GSTP1 function in a simpler *in vitro* system may lead to the identification and detailing of novel mechanistic pathways. To date, *in vitro* studies have yielded a diverse set of possible mechanisms by which GSTP1 may regulate cell function. However, the majority of these are either not translatable to all environments or have not been studied in great detail. This chapter intends to examine cell specific regulation of GSTP1 in an *in vitro* system to uncover novel pathways and processes GSTP1 may be involved in. In particular, this chapter will examine the role of GSTP1 in catalysing a novel posttranslational modification known as protein S-glutathionylation.

4.1 Thiol modification

Modulation of protein thiols is recognised as an important component of protein regulation and sulfhydryl homeostasis. The human proteome codes for around 214,000 cysteine residues, and is relatively underrepresented in most organisms, with the percentage of cysteine residues increasing with the complexity of the organism (Miseta and Csutora, 2000).

Cysteine residues can undergo a number of modifications which may lead to diverse changes in protein function, signalling, folding and localisation, particularly in environments of oxidative stress. This is because thiols have great reducing potential and are strong nucleophiles. In the presence of ROS or RNS, reactive thiols are more likely to donate electrons to radical species, resulting in disulphide modification of thiyl radicals. Thiol groups can undergo a number of modifications, the reactivity of which is dependent on the pKa of the cysteine. Cysteine residues typically have a pKa value of around 8.5 in reducing conditions, such as the cytoplasm, and are unlikely to undergo any modification. Lowering of the pKa value enhances the reactivity of cysteine groups and is influenced by the charge of neighbouring amino acids (Rhee et al., 2000). Electrostatic interactions with basic (positively charged) residues results in a lowering of the pKa and increases the activity of the cysteine group, rendering them more susceptible to posttranslational modifications. Metal binding of cysteine groups, such as zinc, increases the reactivity and susceptibility to oxidation of thiols, an important feature in the regulation of transcription factors (Chen et al., 2004, Zdanowski et al., 2006).

Protection of protein thiols is critical in preventing irreversible oxidation of cysteine residues and loss of protein function. Reaction of thiols with ROS or RNS can lead to a number of intra- and inter-molecular adaptations but commonly leads to the formation of protein-sulfenic acids. Sulfenic acids are unstable and transient in nature (Saurin et al., 2004), often oxidised to sulfinic or sulfonic acids, which are relatively stable and generally irreversible. Alternatively, sulfenic acids can interact with other thiol groups forming intra- and inter-molecular disulphide bridges and can act as intermediates to other thiol modification such as modulation by GSH to form S-glutathionylated proteins (Barrett et al., 1999) which subsequently restores the thiolate anion and acts as a reversible form of thiol protection

against oxidative stress. Although regulation of thiol groups is controlled extensively by a range of posttranslational modifications such as that by the gasotransmitters nitric oxide (van der Vliet et al., 1998) and hydrogen sulphide (Peshenko and Shichi, 2001), this chapter will predominantly focus on the regulation of thiol groups by S-glutathionylation.

4.2 Glutathione

4.2.1 Synthesis and degradation

In its reduced form, the tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is one of the most ubiquitously abundant antioxidants in the cell. Composed of glutamate, cysteine and glycine, GSH is maintained at stable and often high concentrations due to the presence of a γ-carboxyl group linking cysteine and N-terminal glutamate that protects GSH from protease degradation, which typically digest α-carboxyl groups of amino acids (Figure 4.1A). GSH is synthesised *de novo* and is dependent on the availability of cysteine and the activity of key catalysing enzymes. Initially, γ-glutamylcysteine is formed from glutamate and cysteine, catalysed by glutamate-cysteine ligase (GCL), the rate-limiting step in glutathione synthesis. GCL is composed of two subunits, a 'heavy' C unit (73kDa) and a 'light' M unit (28kDa) which are upregulated during oxidative stress through binding of Nrf2 to an antioxidant response element (ARE) in the promoter regions of *GCLC* and *GCLM* (Zhang et al., 2007, Erickson et al., 2002, Wild et al., 1999). The C subunit possesses the catalytic activity for GCL while the M subunit is thought to enhance the efficiency of the enzyme and reduce the sensitivity to feedback inhibition (Tu and Anders, 1998, Choi et al., 2000). Glutathione synthetase (GS) adds glycine to the γ-glutamylcysteine unit to form GSH. GSH synthesis from cysteine can also be produced from the catabolism of methionine via the transsulfuration pathway but is only specific to hepatocytes (Finkelstein, 1990).

GSH is specifically hydrolysed by the ectoenzyme γ-glutamyltranspeptidase (GGT) upon its transport from the cell. GSH can be exported in its reduced or disulphide form and as a conjugate. Extracellular GSH is hydrolysed in an ATP dependent manner at the gamma linkage between glutamate and cysteine releasing cysteinylglycine and glutamate, which in turn is transferred to another amino acid, often cystine. Cysteinylglycine is cleaved by dipeptidase into cysteine and glycine which are then transported back into the cell. Glutamyl conjugates are also transported into the cell where the glutamyl residue forms 5-oxoproline and then glutamate, through the action of 5-oxoprolinase. Cystine can also be transported into the cell or via conjugation with glutamyl where it is reduced to cysteine. In this regard, GSH acts as a reservoir for cysteine storage, as cysteine is unstable extracellularly as it is often oxidised to cystine. Control of cysteine/cystine redox potential in extracellular compartments is important in maintaining redox homeostasis, changes in which can lead to activation of intracellular free radicals and upregulation of glutamate receptors (Zhu et al., 2012). Effective uptake of cysteine/cystine has been shown to be important in cancer cell survival. In a recent study by Zhang et al, bone marrow stromal cells were found to enhance the survival of chronic lymphocytic leukaemia (CLL) cells through interactions of the cysteine/cystine transport system, despite inefficient uptake of cystine by CLL cells due to low expression of the cystine transporter Xc^- (Zhang et al., 2012). It was hypothesised that stromal cells import cystine and convert it to cysteine which is released into the microenvironment and subsequently taken up by CLL cells. Inhibition of cystine uptake by stromal cells significantly decreased GSH levels in CLL cells and leads to cell death demonstrating a role of GSH in cancer cell survival.

In most cellular organelles, particular the cytoplasm and mitochondria, about 90-99% of GSH is present in its reduced form to maintain cellular function and redox homeostasis. However, in the endoplasmic reticulum, the ratio of reduced and disulphide glutathione is more balanced as disulphide bonding is necessary for the proper folding of proteins during synthesis. GSH is synthesised only in the cytoplasm, where it is then transported to other organelles. Typically, around 10% of GSH is localised in the mitochondria. Interestingly, mitochondria do not appear to efflux oxidised glutathione and reduction of disulphide to reduced glutathione is mediated predominantly by glutathione reductase (Olafsdottir and Reed, 1988). As mitochondria contain little volume, the concentration of GSH relative to the cytoplasm is greater and therefore has a lower reduction potential (Go and Jones, 2008). To overcome this gradient, two mitochondrial transporters, the 2-oxoglutarate and dicarboxylate carriers aid in GSH transport and account for around 80% of total GSH transport into the mitochondria (Lash et al., 2002, Chen et al., 2000b). The mitochondrial glutathione reduction potential is of great importance in relation to protection against free radicals, as it is estimated that the majority of cellular ROS are associated with the mitochondria (Balaban et al., 2005).

4.2.2 Role as an antioxidant

Radical intermediates formed from oxidative reactions such as superoxide ($O_2^{\cdot-}$), nitric oxide ($^{\cdot}NO$) and hydrogen peroxide (H_2O_2) contribute to cell injury through direct oxidation of proteins, DNA and lipids or by inducing apoptotic and necrotic pathways. GSH serves as a reducing agent within the cell and has a central role in maintaining redox homeostasis through a diverse set of mechanisms such as conjugating reactive metabolites, maintaining the thiol redox status of proteins, iron transfer and modulating cellular processes. Antioxidant reactions involving GSH are mediated through a reactive sulfhydryl present on the cysteine residue where it conjugates electrophiles spontaneously or is catalysed enzymatically via

glutathione S-transferases. GSH can also mediate the reduction of peroxides, acting as a co-substrate for glutathione peroxidase in the degradation of hydrogen peroxide. In addition, many endogenous compounds such as prostaglandins and leukotrienes and endogenously produced toxicants such as 2-oxopropanal and lipid peroxidation metabolites, are detoxified by GSH, either directly or acting as a cofactor (Heasley and Brunton, 1985, Rouzer et al., 1981, Inagi et al., 2010). GSH also mediates the cellular export of a number of xenobiotic compounds via the multidrug-resistance-associated protein (MRP) and there have been numerous studies detailing high concentrations of GSH have been associated with resistance to anticancer compounds and cell survival (Zaman et al., 1995, Mulcahy et al., 1994). Reaction of GSH with radical species leads to the production of thiyl radicals ($GS^{\cdot}$) which in turn can form disulphide bonds with other molecules or dismutate with other glutathionyl anions resulting in glutathione disulphide (also known as oxidised glutathione, GSSG, Figure 4.1B). Perturbations in cellular redox levels as a result of oxidative stress and increased ROS production leads to increased levels of glutathione disulphide formation (Jones, 2006). Once formed, GSSG can be exported from the cell or reduced back to GSH by glutathione reductase at the expense of NADPH.

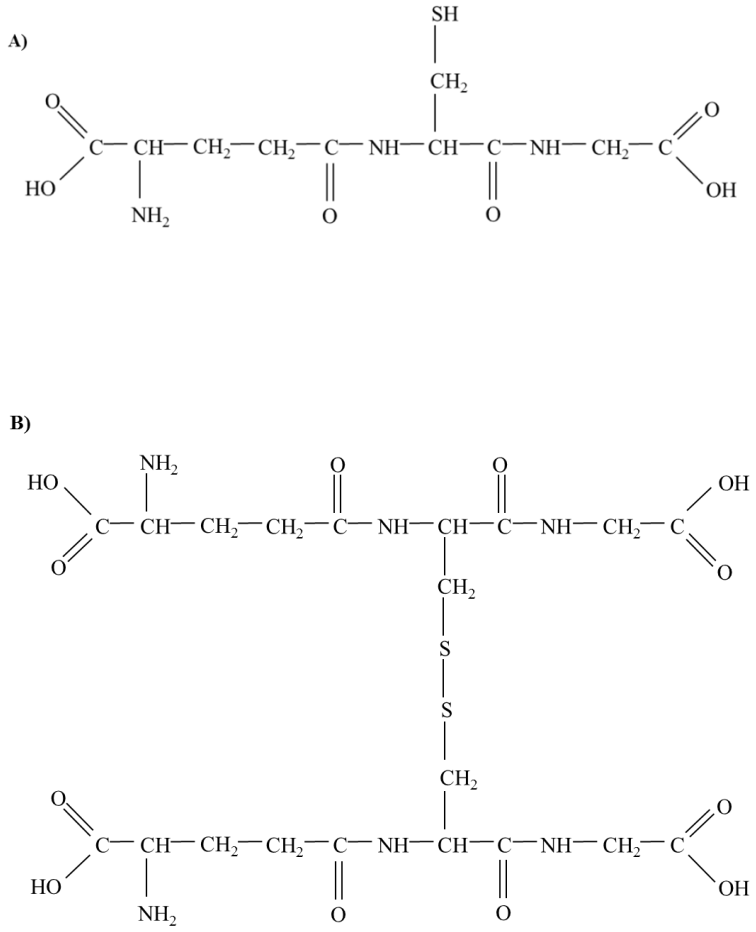


Figure 4.1. Molecular structures of glutathione in its A) reduced form and B) disulphide form.

4.3 Protein S-glutathionylation

The function of glutathione extends well beyond its role as an antioxidant, possessing many properties related to cell signalling. One mechanism by which GSH is thought to regulate cell function is through conjugation with protein thiols. Protein S-glutathionylation is a reversible posttranslational modification involved in sulfhydryl homeostasis, believed to protect protein thiols against irreversible oxidation. In S-glutathionylation, the glutathione anion ($GS^{\cdot}$) forms a disulphide bond with reactive protein thiols preventing oxidation of cysteine residues from ROS/RNS and oxidative metabolites derived from carbohydrate and lipid modification

(Refsgaard et al., 2000). Protein S-glutathionylation can be induced globally (Eaton et al., 2002) or selectively (Adachi et al., 2004) depending on the origin of oxidative stress but can also be found at resting levels (Wang et al., 2001a), suggesting that S-glutathionylation may play a functional role in protein regulation as well as thiol protection. Conversely, protein S-glutathionylation may also function in preventing the export of GSH from the cell in the form of GSSG, concentrations of which are increased and exported from the cell under oxidative stress. The function of protein S-glutathionylation *in vivo* is still not fully understood, induction of which is prevalent in the aetiology of certain diseases related to oxidative stress such as diabetes (Sampathkumar et al., 2005), cardiovascular disease (Eaton et al., 2002), Parkinson's (Naoi et al., 2008) and Alzheimer's (Di Domenico et al., 2009). Whether protein S-glutathionylation confers a difference in protein function contributing to a particular disease or is a direct consequence of oxidative stress (and therefore has potential as a therapeutic biomarker) is still to be fully addressed.

4.3.1 Mechanisms of protein S-glutathionylation

Proteins can become S-glutathionylated through a diverse set of pathways. Common mechanisms resulting in protein S-glutathionylation often involve the production of a reactive intermediate of cysteine modification such as sulfenic acids (Barrett et al., 1999), thiyl radicals (Starke et al., 2003) or thiosulfinate intermediates (Li et al., 2001), all of which are relatively unstable and are highly reactive with thiol groups. Nitric oxide (NO) mediated S-nitrosylation of glutathione (S-nitrosoglutathione, GSNO) and proteins (S-nitrosothiols, PSNO), although stable, may also act as an intermediate to protein S-glutathionylation. GSNO can induce the S-glutathionylation of a number of proteins *in vitro* (Giustarini et al., 2005, Mohr et al., 1999). However, due to the stability of GSNO it is unclear what conditions favour protein S-glutathionylation over PSNO *in vivo* although it is hypothesised that

neighbouring amino acid groups may influence the outcome of a cysteine becoming S-nitrosylated or S-glutathionylated (Giustarini et al., 2005). Although reactive intermediates from cysteine modifications appears to be a more likely mechanism for protein S-glutathionylation *in vitro,* there is still very little evidence that the kinetic and thermodynamic biochemistry of these reactions are upheld *in vivo*.

One of the more hypothetical mechanisms of protein S-glutathionylation is through disulphide-exchange of the protein thiols. GSH is largely reduced in the cell but under oxidative conditions, dismutates to form GSSG. Hypothetically, GSSG accumulation could induce protein S-glutathionylation through a thiol-disulphide exchange between a protein thiol and GSSG as a means of regulating cellular redox levels and acting as a form of GSH storage within the cell. However, many observers have deemed this route improbable *in vivo* predominantly because the ratio of GSH to GSSG would have to drop dramatically to shift the disulphide equilibrium towards protein thiolation (Gilbert, 1995), while some have questioned the accessibility of GSSG to sulfhydryl groups due to its increased mass and charge (Hu et al., 2010). However, there is evidence to suggest that some proteins which have atypical redox potentials such as c-Jun may be susceptible to S-glutathionylation through disulphide exchange at high GSH:GSSG concentrations (Klatt et al., 1999).

4.3.2 Glutaredoxins

Protein S-glutathionylation can be reversed through the action of glutaredoxin, which possesses high specificity for S-glutathionylated thiols over other disulphide modifications. Two isoforms largely account for the majority of deglutathionylation reactions; Grx1 which is localised to the cytosol, and Grx2 which is localised in the mitochondrial matrix. Grx1

contains a CPYC motif which contains an exposed Cys11 residue (Bushweller et al., 1992), while Grx2 contains a CSYC within its active site and a Cys70 residue which is involved in deglutathionylation (Gladyshev et al., 2001). GSH is transferred from the protein to the Cys residue which can then react with another GSH molecule to yield GSSG. Recent *in vivo* studies have highlighted the importance of Grx1 in mediating deglutathionylation of cellular proteins. Hoffman et al demonstrated that *Grx1*$^{-/-}$ mice have an attenuated airway hyper-responsiveness to ovalbumin which correlates with an increase in protein S-glutathionylation as well as a decrease in other inflammatory mediators (Hoffman et al., 2012), while Wu et al showed *Grx2*$^{-/-}$ mice to have increased sensitivity to oxidative stress and increased protein S-glutathionylation (Wu et al., 2011). Sulforedoxin, an oxidoreductase, has also been suggested as an enzyme involved in the deglutathionylation of cellular proteins, however it is more likely that it reduces the intermediates formed prior to protein S-glutathionylation than S-glutathionylated proteins themselves.

4.3.3 Identification and function of S-glutathionylated proteins in biology

Due to the influence of the local protein micro-environment on cysteine reactivity, there does not appear to be a canonical sequence motif for reactive cysteine residues within proteins which makes identification and characterisation of susceptible proteins to posttranslational modification difficult. Weerapana et al attempted to characterise cysteine functionality using a large scale quantitative proteomic approach encompassing LC-MS/MS analysis of cysteine residues labelled with an alkynylated iodoacetamide (IAM) probe (Weerapana et al., 2010). This approach quantitated the reactivity of IAM labelled cysteines, and therefore identifies cysteine residues susceptible to general posttranslational modification. In relation to S-glutathionylation, a number of proteins have been identified as S-glutathionylated using an array of biochemical methods (Table 7). The impact of S-glutathionylation on cellular

proteins is somewhat contradictory. There is a large body of evidence that demonstrates in times of oxidative stress, S-glutathionylation appears to inhibit protein function. For example, S-glutathionylation of p53 reduces its ability to bind to DNA (Velu et al., 2007) while S-glutathionylation of actin reduces its affinity for tropomyosin and therefore reduces its ability to polymerise (Chen and Ogut, 2006). In contrast, ROS induction following Angiotensin II treatment of smooth muscle cells causes S-glutathionylation of Ras which subsequently leads to increased p38 and Akt phosphorylation (Adachi et al., 2004). It is interesting to note that GSTP can also be S-glutathionylated at 2 cysteine residues, Cys47 and Cys101 which affect its apparent binding to target proteins (Townsend et al., 2008a, Tew et al., 2011). This is particularly interesting with regards to a potential role of GSTP in the catalysis of protein S-glutathionylation.

Protein class	Impact of glutathionylation	Notable proteins of interest
Proteins with thiol active centres	In general, S-glutathionylation results in function inhibition. S-glutathionylation of PrdxVI results in reactivation of enzyme.	Carbonic anhydrase III, Tyrosine hydrolase, α-ketoglutarate dehydrogenase, aldose reductase, creatine kinase, GAPDH, HIV-1 protease, PrdxI, PrdxVI, Inosine 5'-monophosphate dehydrogenase 2, protein disulphide isomerase, elonase 1α, phosphoglycerate kinase, aldolase, phosphorylase kinase, 6-Phosphogluconolactonase, Triosephosphate isomerase, Adenylate kinase 2, dUTP pyrophosphatase, Peptidylprolyl isomerase (cyclophilin A), Cytochrome c oxidase, Ubiquitin-conjugating enzyme E2N, Thioredoxin 1 Glutathione S-transferase P1, Endothelial nitric oxide synthase
Cytoskeletal proteins	S-glutathionylation results in function inhibition or inhibition of polymerisation/filament formation.	Vimentin, G-Actin Tropomyosin, Transgelin, SM22 homolog calponin-like, Cofilin, Myosin, Profilin, Beta-Tubulin, Annexin II, Spectrin
Signalling proteins	In general, S-glutathionylation results in function inhibition. S-glutathionylation of T cell $p59^{fyn}$ kinase and p21 ras results in function activation.	Protein kinase A, Protein kinase C, ERK, T cell $p59^{fyn}$ kinase, PTP1B, MEKK1, PTEN, Protein kinase G, c-Abl, Caspase 3, p21 ras
Transcription factors	S-glutathionylation results in function inhibition.	c-Jun, NF-κB subunits 65 and 50, IKK β-subunit, Pax-8, OxyR, p53
Heat shock	S-glutathionylation results	HSP60, HSP70

proteins	in function inhibition.	
Ion channels, transport pumps	In general, S-glutathionylation results in function activation. S-glutathionylation of CFTR results in function inhibition.	RyR1, CFTR, SERCA, S100 A1, S100 A4, S100 B
Energy metabolism/glycolysis	S-glutathionylation results in function inhibition.	Complex I, NADP+-dependent isocitrate dehydrogenase, Cytochrome oxidase, ATPase, NADH ubiquinone reductase, Carbonic anhydrase III, Catechol-O-methyltransferase, Pyruvate dehydrogenase

Table 7. Overview of S-glutathionylation on protein class and function.

The table highlights a brief overview on the number of proteins which are thought to be S-glutathionylated and the biological impact of S-glutathionylation on protein function. Adapted from Tew et al 2011.

4.3.4 GSTP1 as a catalyst of protein S-glutathionylation

S-glutathionylation is often regarded as a transient reaction in response to oxidative stress. However, some groups have recently argued that the reaction may be catalysed by GSTP1. Peroxiredoxins (Prdxs) reduce hydroperoxides to alcohols via active cysteine residues. There are two main families of peroxiredoxins; those containing two conserved cysteine residues in their C-terminal domain (2-Cys) and those only containing one cysteine residue (1-Cys). The lack of an extra cysteine molecule for the 1-Cys family, or peroxiredoxin VI (PrdxVI), means that another molecule containing a thiol group is needed in order to reduce the oxidised Cys 47 residue and to regenerate the active PrdxVI (Choi et al., 1998). It has been suggested that GSTP1 provides the thiol group required in a glutathionylation step by forming a heterodimer

with PrdxVI (Manevich et al., 2004) and it has been demonstrated *in vitro* that a stable GSTP1:Cys1 complex could be formed which restores active PrdxVI activity (Ralat et al., 2006). However, due to the large number of inter-disulphide interchange reactions, the kinetic competence of this mechanism has been questioned (Gallogly and Mieyal, 2007).

Further evidence for GSTP1-mediated S-glutathionylation has been proposed from the laboratory of Professor Kenneth Tew. Townsend et al neatly demonstrated that the nitric oxide releasing prodrug PABA/NO results in apoptosis and protein kinase activation correlating with increased protein S-glutathionylation and the presence of GSTP1 (Townsend et al., 2006). The group later demonstrated that global protein S-glutathionylation was potentiated by GSTP1 in response to nitrosative and oxidative stress (Townsend et al., 2008a). Whether this is due to the specificity of GSTP1 for PABA/NO or the increased cytotoxic effect of PABA/NO on GSTP1 expressing cells is yet to be determined. It appears contradictory that GSTP1 can catalyse protein S-glutathionylation in times of oxidative and nitrosative stress, when itself is S-glutathionylated leading to reduced activity. The same group has recently collaborated on a number of publications demonstrating that GSTP1 may potentiate the S-glutathionylation of the death receptor Fas (Anathy et al., 2012) while showing a potential role of GSTP1 in controlling cocaine induced protein S-glutathionylation (Uys et al., 2011).

The role of GSTP1 in mediating protein S-glutathionylation is still not fully understood. Nonetheless, the ability of GSTP1 to conjugate GSH to many xenobiotic substrates makes it a potential candidate in catalysing GSH conjugation to proteins. The focus of this chapter will be to examine the extent of protein S-glutathionylation mediated by GSTP1 *in vitro*.

Results

4.4 Transient silencing of GSTP1 in HCT116 cells

The human colon carcinoma cell line, HCT116, was used in this study as they are near diploid, stable in cell culture and, have been shown to require GSTP1 expression for proliferation, demonstrating a potential role for GSTP1 in mediating cellular function independent of drug metabolism (Dang et al., 2005). GSTP1 was transiently silenced in HCT116 cells using siRNA targeted against GSTP1 or a control siRNA to account for off target effects. Cells were also mock transfected with nuclease free water to account for off target effects associated with siRNA transfection. The concentration of siRNA to be used was optimised by transfecting increasing concentrations of siRNA (from 1nM to 50nM) into HCT116 cells and determining GSTP1 expression from 24 to 72 hours post transfection. The minimal concentration of siRNA which resulted in maximal depletion of GSTP1 protein was found to be 10nM after 72 hours, as determined by Western blot analysis (data not shown).

Figure 4.2 demonstrates that transient knockdown of GSTP1 in HCT116 cells does not induce compensatory changes in other GSTs or antioxidant response genes, as shown by Western blotting of GSTM1 and NAD(P)H: quinone oxidoreductase 1 (NQO1) expression. NQO1 catalyses the detoxification of quinones and is induced in response to a number of carcinogenic and pro-oxidant compounds. The lack of NQO1 induction as a result of GSTP1 silencing suggests that the cellular redox state does not appear to be intrinsically regulated by GSTP1 in HCT116 cells.

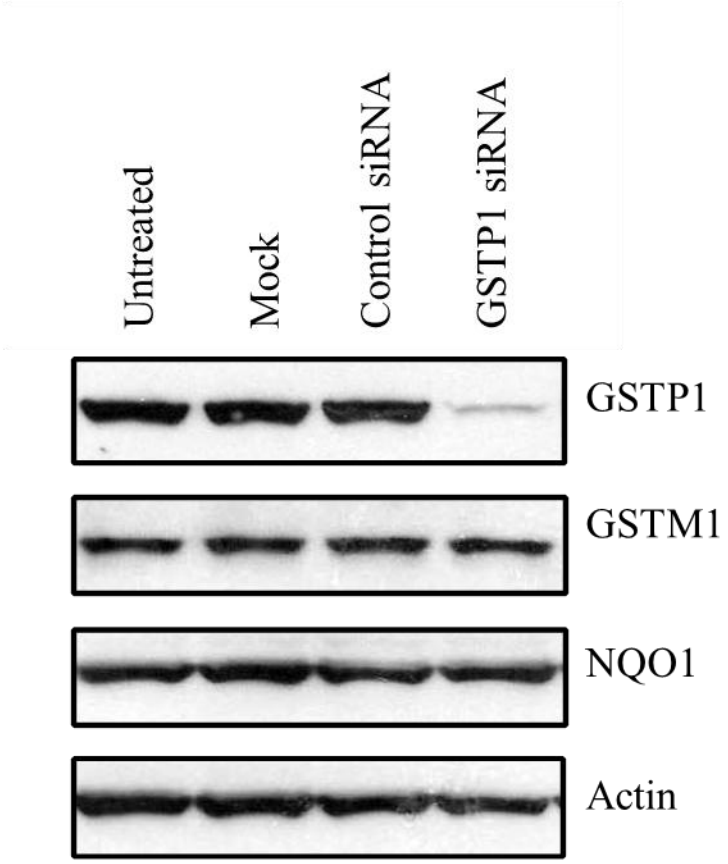


Figure 4.2. Transient silencing of GSTP1 in HCT116 cells.

HCT116 cells were transiently transfected with nuclease free water (mock), 10nM siRNA targeted against GSTP1 or control siRNA. After 72 hours, cells were harvested and lysates (10µg) resolved on a SDS-PAGE gel before analysed by Western blotting. Blots are representative of 3 independent experiments.

This observation is strengthened further through examination of the cellular morphology. Silencing of GSTP1 does not appear to induce any significant change in cellular morphology and the cells show no apparent signs of stress or apoptosis as a result of GSTP1 silencing (Figure 4.3). The only noticeable difference is the apparent protruding outer membranes of a number of cells silenced for GSTP1. This phenotype may be related to changes in cytoskeletal expression which are discussed later in Chapter 5. It is important to address that while the lack of noticeable changes in morphology and NQO1 induction may imply that redox and other cellular changes have not occurred, upregulation of other compensatory pathways that have not yet been accounted for may be implicit in maintaining cellular homeostasis.

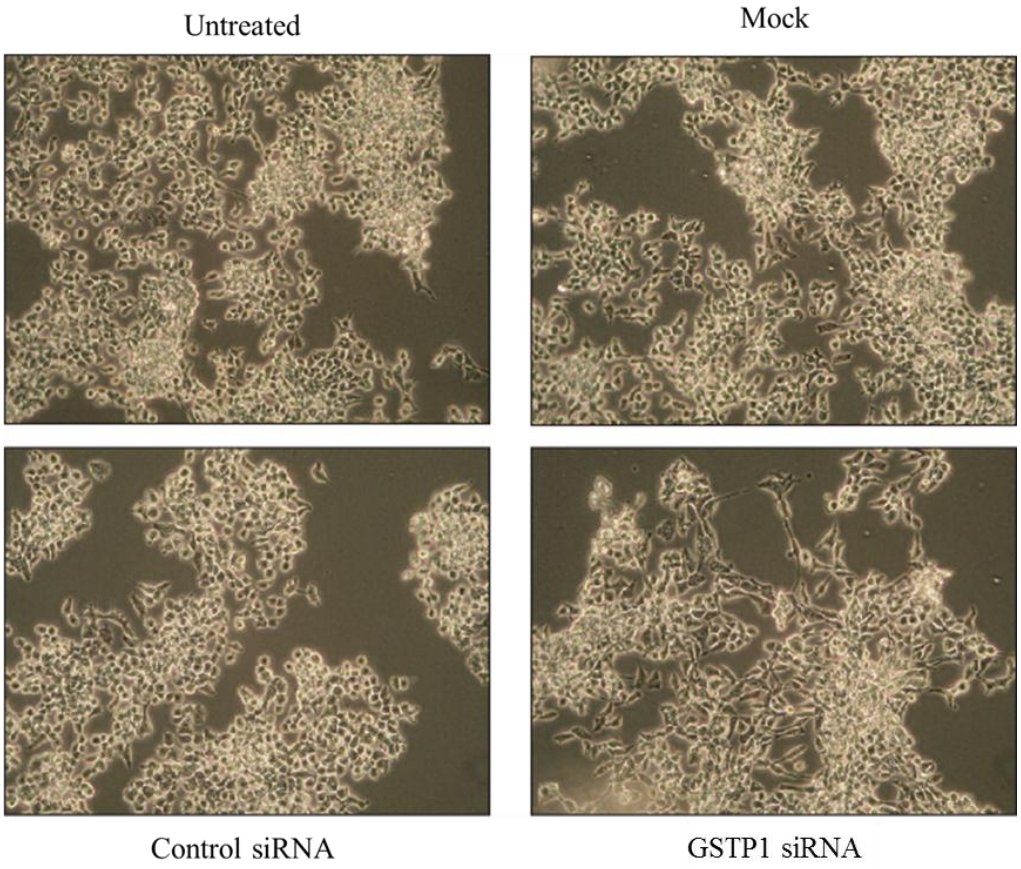


Figure 4.3. Examination of the cellular morphology of GSTP1 silenced HCT116 cells.

HCT116 cells were transiently transfected with nuclease free water (mock), 10nM siRNA targeted against GSTP1 or control siRNA. The morphology of the cells was examined 72 hours post transfection. Pictures are representative of 3 independent experiments.

4.5 Role of GSTP1 in proliferation of HCT116 cells

In the initial characterisation of HCT116 cells, the extent to which GSTP1 can affect cell cycle and survival was examined through cell cycle profiling of HCT116 cells. Cells were transfected with GSTP1 siRNA, stained with propidium iodide (PI) and analysed by flow cytometry. HCT116 cells were transfected with 10nM of control siRNA or siRNA targeted against GSTP1 and stained with PI 72 hours post transfection as described under 'Materials and Methods'. In line with morphological observations, examination of the cell cycle profile of HCT116 cells silenced for GSTP1 staining demonstrates little change in apoptosis or arrest as shown by low levels of G1/G0 (subG1) (Figure 4.4A). Detailed analysis of apoptotic pathways using the Intellicyt Multimetric Apoptosis Screening kit demonstrated no change in caspase activation, annexin V, mitochondrial integrity or cell viability (Figure 4.4B). Silencing of GSTP1 increases the proportion of cells in G1, suggesting that they are spending more time in the 'growth phase' of the cell cycle and are slower at progressing through into mitosis, indicative of the lower S and G2/M phases also shown. This observation correlates neatly with an increase in doubling time of HCT116 cells silenced for GSTP1. Although not always statistically significant, a trend in cells silenced for GSTP1 demonstrates a longer doubling time than their wild-type or control treated counterparts when seeded at different densities (Table 8). As GSTP1 silenced cells are remaining in G1 for longer periods, this decreases the number of cells undergoing mitosis and therefore leads to a reduction in cell proliferation. Although GSTP1 is not essential for the prevention of cell cycle arrest, the results demonstrates that GSTP1 is important for the proliferation of HCT116 cells in culture. The precise mechanism by which this occurs is not fully understood, however differences in cytoskeleton remodelling as a result of GSTP1 knockdown may contribute to the reduction in cell proliferation and is demonstrated and discussed in Chapter 5.

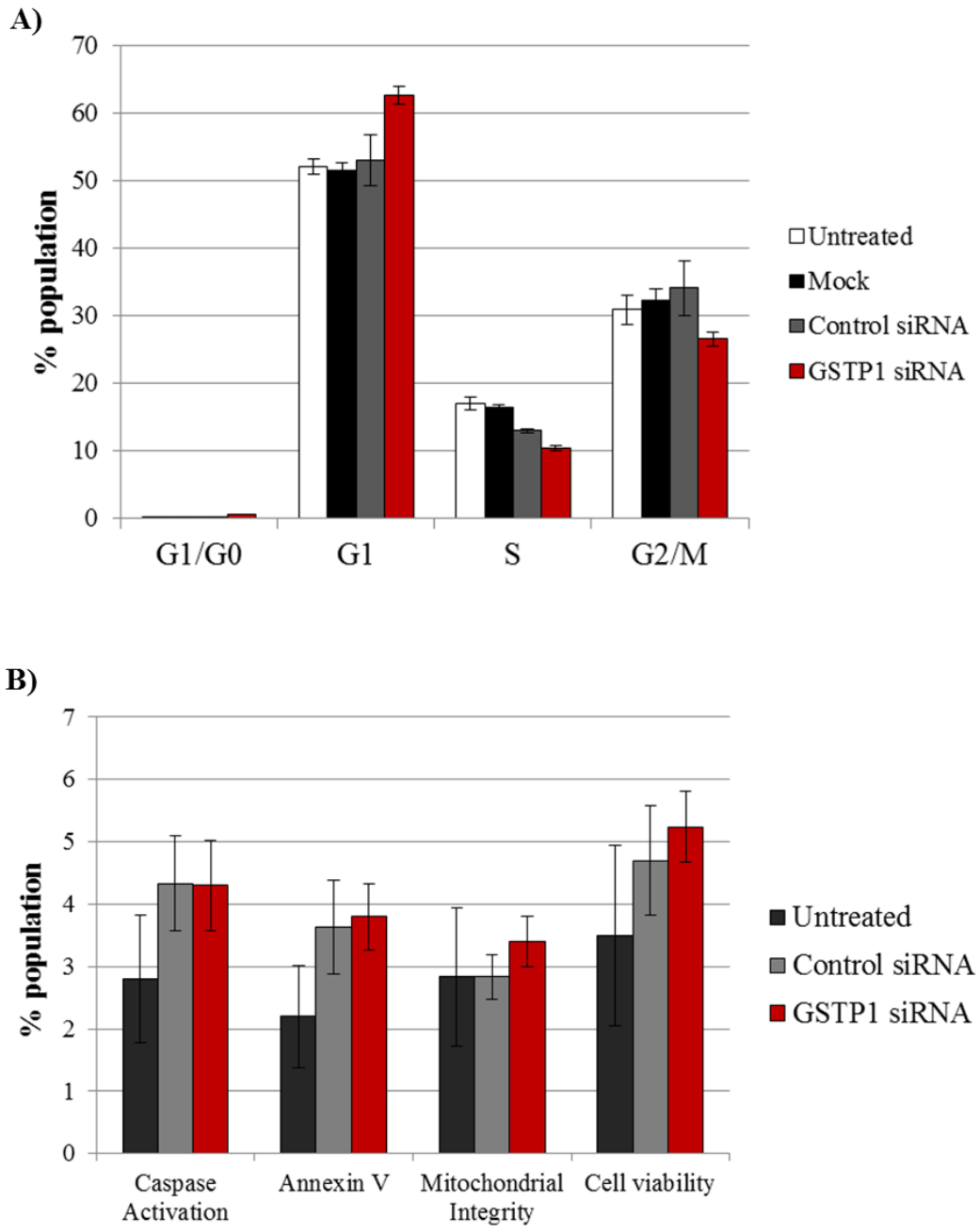


Figure 4.4. Cell cycle profile of GSTP1 silenced HCT116 cells.

HCT116 cells were transiently transfected with nuclease free water (mock), 10nM siRNA targeted against GSTP1 or control siRNA (n=3). **(A)** After 72 hours, cells were washed and fixed in 70% ethanol before stained with propidium iodide. Cell cycle profiles were analysed by flow cytometry. **(B)** After 72 hours, cells were washed and stained for a number of apoptotic pathways using the Intellicyt Multimetric Apoptosis Screening kit. Apoptotic parameters were measured simultaneously using a Becton Dickinson LSRFortessa. Values are represented as mean ± standard deviation.

	Doubling time of HCT116 cells after siRNA treatment (hrs)		
Number cells seeded per well	Untreated	Control siRNA	GSTP1 siRNA
2000	17.32±3.35	20.53±0.88	25.69±1.49
1000	17.42±0.95	23.51±2.93	23.36±1.75
500	19.41±3.22	21.88±1.54	29.73±3.00*$
250	18.78±3.91	20.28±1.05	26.08±7.35

Table 8. Doubling time (hrs) of GSTP1 silenced HCT116 cells.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA. Post transfection (24 hours), cells were trypsinised and seeded onto a 96 well plate at the seeding densities described in the table. The doubling time was assessed 72 hours after seeding using the ATP assay. To establish that ATP levels correlate with cell number and not differences in metabolic pathways, preliminary studies determined cell number using trypan blue staining and the number of cells was found to strongly correlate with ATP levels. Values are represented as mean ± standard deviation, where * GSTP1 vs Untreated $P < 0.05$, $ GSTP1 vs control siRNA $P < 0.05$.

4.6 GSTP1 mediated cell regulation of JNK inhibition

As described in Chapter 3, one of the proposed mechanisms by which GSTP1 is thought to mediate cell signalling is through inhibition of JNK activity. Under certain stresses, GSTP1 inhibition of JNK prevents the downstream phosphorylation of its transcription factor c-Jun and subsequent transcription of stress response genes (Adler et al., 1999). This model suggests that in the absence of GSTP1, JNK activity is propagated under basal and stress conditions, leading to increased stress response. In order to examine this hypothesis, HCT116 cells were silenced for GSTP1 using siRNA as described previously before exposed to

different doses of UV radiation. Cells were incubated for 1 hour at 37°C in culture media after UV exposure before lysates were prepared and resolved on a SDS-PAGE gel. Figure 4.5 demonstrates that exposing HCT116 cells to increasing levels of UV increases the level of JNK phosphorylation and phosphorylation of its downstream target, c-Jun. However, this figure clearly demonstrates that there is no difference in the level of JNK phosphorylation in the presence or absence of GSTP1, highlighting that in HCT116 cells, GSTP1 must have other cellular functions besides regulation of JNK activity.

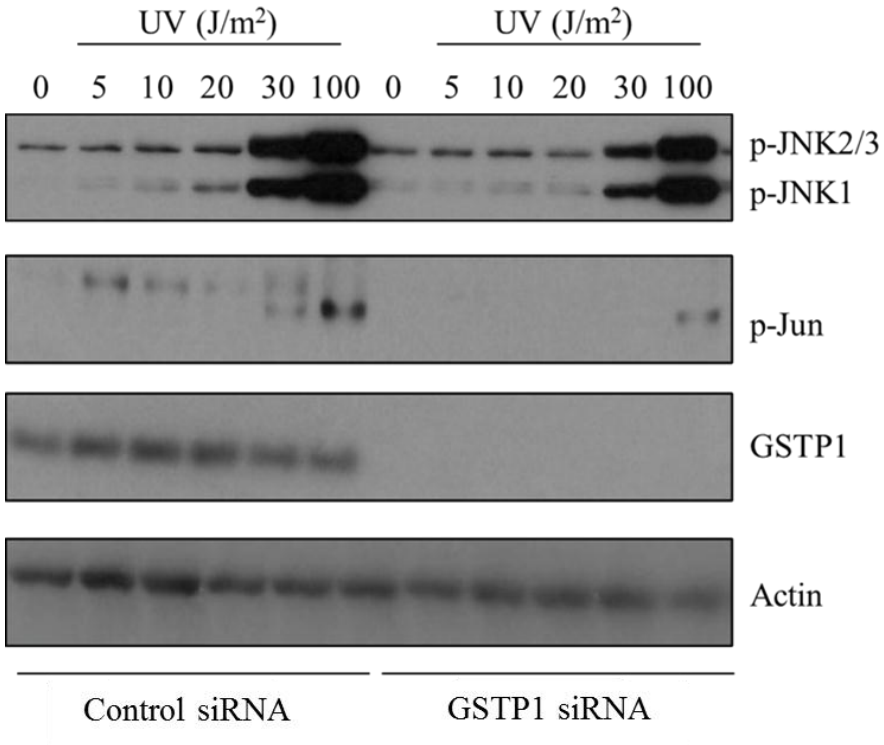


Figure 4.5. UV induction of JNK phosphorylation is not attenuated in the presence of GSTP1 in HCT116 cells.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA and grown for 72 hours. HCT116 cells were exposed to increasing doses of UV radiation and incubated in media for 1 hour at 37°C. Cells were harvested and lysates (10µg) were resolved on a SDS-PAGE gel and analysed by Western blotting. Blots are representative of 3 independent experiments.

4.7 Oxidation state of HCT116 cells silenced for GSTP1

GSTP has previously been shown to be an important mediator in redox control (Townsend et al., 2006). To determine the amount of reactive oxygen species produced as a result of silencing GSTP1, HCT116 cells were incubated with dichlorodihydrofluorescein diacetate (H_2DCFDA), a reduced fluorescein which is readily converted to a green-fluorescent form in the presence of oxidation. Silencing of GSTP1 does not appear to induce the level of ROS within HCT116 cells as we cannot detect any shift in H_2DCFDA intensity as determined by flow cytometry (Figure 4.6). Therefore at resting levels within HCT116 cells, the production of reactive intermediates is not dependent on the function of GSTP1. However, that may be dependent on increases in other redox mechanisms compensating for the scavenging of excess ROS intermediates as a result of silencing GSTP1.

One of the most abundant radical scavengers in the cell is the tripeptide glutathione. Examination of total and disulphide glutathione levels may indicate whether silencing of GSTP1 induces changes in the redox status of HCT116 cells. The levels of total and oxidised glutathione were analysed in HCT116 cells 72 hours following transient knockdown of GSTP1 using siRNA. As Figure 4.7 demonstrates, transient silencing of GSTP1 increases the total concentration of glutathione within the cell (Figure 4.7A) and increases the proportion of glutathione which is oxidised (Figure 4.7B), suggesting an elevation in ROS/oxidative stress formation in HCT116 cells after silencing GSTP1.

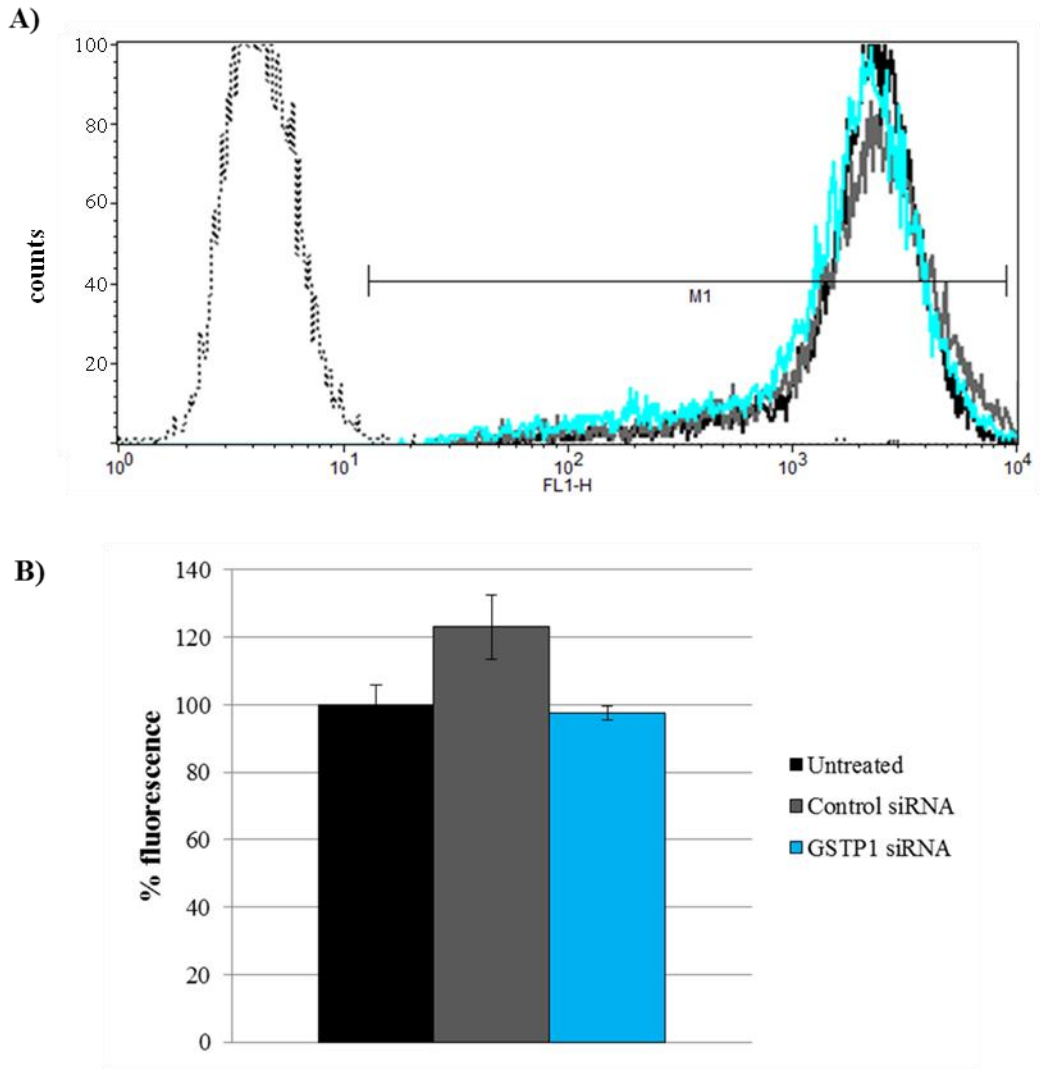


Figure 4.6. Dichlorodihydrofluorescein diacetate (H_2DCFDA) staining of GSTP1 silenced HCT116 cells.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA (n=3). After 72 hours, cells were incubated with 10µM H_2DCFDA in PBS for 30 minutes. Cells were trypsinised, washed in PBS and analysed by flow cytometry. **A)** Histogram plot of HCT116 cells treated with H_2DCFDA. The left hand peak is cells treated with PBS only and used as a negative control against H_2DCFDA staining. M1 denotes area of shift from PBS control samples**. B)** The bar chart represents the area under each histogram plot. Fluorescence is shown as a percentage of untreated HCT116 cells. Values are represented as mean ± standard deviation.

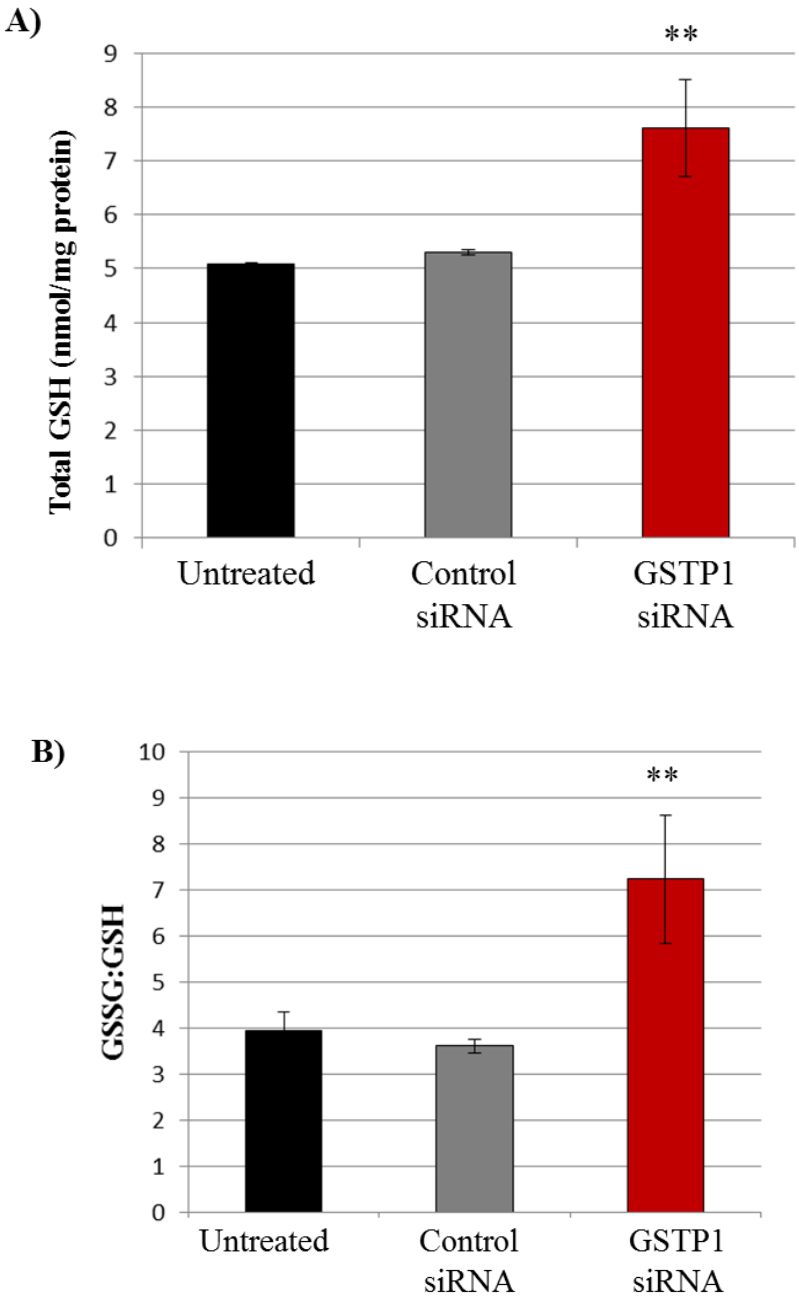


Figure 4.7. Glutathione levels in GSTP1 silenced HCT116 cells.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA (n=3). Levels of total **(A)** and oxidised **(B)** glutathione were analysed 72 hours after siRNA transfection. Values are represented as mean ± standard deviation, where ** P <0.01 compared to untreated and control siRNA values.

4.8 Role of GSTP1 in the protein S-glutathionylation of HCT116 cells

The function of glutathione within the cell has broadened from that of a metabolite scavenger. Protein S-glutathionylation is a reversible post-translational modification in which proteins are conjugated to glutathione in what is thought to be a protective function against irreversible oxidative stress. It has been proposed that GSTP1 can potentiate the S-glutathionylation of a number of proteins following oxidative and nitrosative stress (Townsend et al., 2008a), although studies have yet to determine whether this is a global event or particular to only a subset of proteins. After observing differences in the levels of total and oxidised glutathione after silencing of GSTP1, the level of protein S-glutathionylation was determined in HCT116 cells using a biotinylated form of glutathione known as biotinylated glutathione ethyl ester (BioGEE). BioGEE is a cell-permeable glutathione analogue which is transiently incorporated into proteins under conditions of oxidative stress and can therefore be used as marker of protein S-glutathionylation. Although other *in vitro* methods have been described, many common procedures rely on the biotin-switch method in which Grx reduction of the S-glutathionylated protein is labelled with another molecule, such as biotin-HPDP. Whilst many proteins have been identified using this method, the false positive rate is relatively high due to the number of procedure steps involved (increasing the chance of oxidative stress not associated with cell culture treatment) and the sensitivity of exposed thiol groups to light. BioGEE also avoids many of the false positives associated with using anti-glutathione antibodies and provides *in situ* labelling of S-glutathionyated proteins without introducing further oxidative stress. The initial hypothesis assumes that if GSTP1 is active in the catalysis of protein S-glutathionylation, HCT116 cells silenced for GSTP1 would show a marked reduction of biotin incorporation when treated with BioGEE.

GSTP1 was transiently silenced in HCT116 cells as described previously. HCT116 cells were treated with BioGEE (0.4mM) for 1 hour, 72 hours post siRNA transfection. Cells were lysed in RIPA buffer supplemented with 25mM N-ethylmaleimide to prevent further modification of thiol groups and lysates were resolved on a non-reducing SDS-PAGE gel and analysed by Western blotting. A streptavidin antibody conjugated to horseradish peroxidase (HRP) was used for the detection of biotinylated proteins. Figure 4.8 demonstrates that BioGEE was incorporated into HCT116 cells and results in the labelling of a number of proteins. There is no difference in the level of streptavidin binding between untreated HCT116 cells and cells transfected with a control siRNA, suggesting that BioGEE was incorporated equally in control cells. However, in GSTP1 silenced cells, streptavidin expression is greatly increased, suggesting more incorporation of BioGEE and therefore higher levels of protein S-glutathionylation when expression of GSTP1 is silenced. HCT116 cells were incubated with BioGEE for 1 hour although other time points have been tested with similar results. Longer time courses do not necessarily result in greater incorporation of the biotin signal, while similar (although slightly weaker) signals can be detected after 30 minutes incubation (data not shown). In contrast to our initial hypothesis, the data imply that GSTP1 is not required for the S-glutathionylation of cellular proteins and silencing GSTP1 in HCT116 cells significantly increases the level of protein S-glutathionylation. Initial results suggest that there is no difference in the number of proteins which are S-glutathionylated, but that these proteins are more susceptible to the labelling of BioGEE.

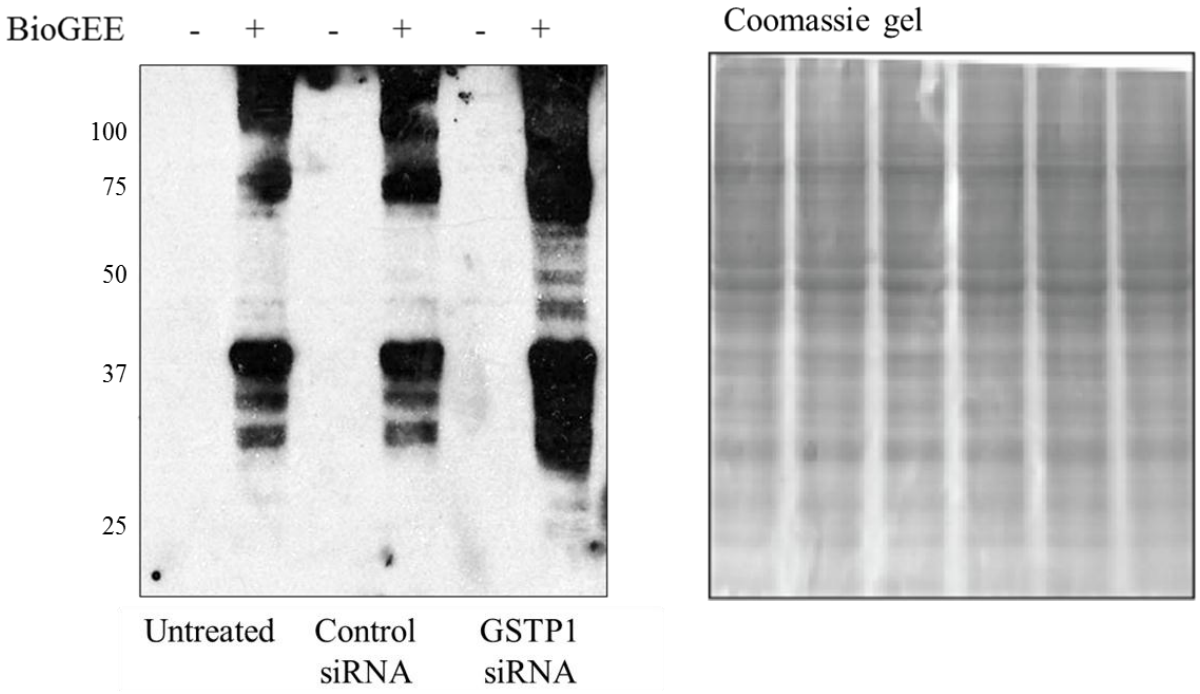


Figure 4.8. Protein S-glutathionylation of HCT116 cells silenced for GSTP1.

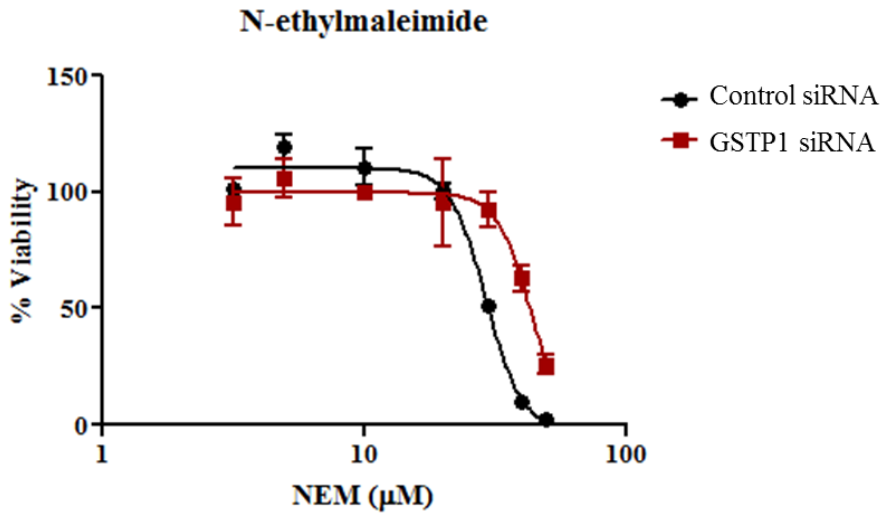
HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA (n=3). Cells were incubated with BioGEE (0.4mM) for 1 hour, 72 hours post siRNA transfection. Cells were harvested and lysates (10µg) resolved on a SDS-PAGE gel in non-reducing conditions and analysed by Western blotting. The Western blot shows the presence of biotinylated proteins as determined by streptavidin-HRP (1:1000), while the coomassie-stained gel demonstrates equal loading of lysates. Blots are representative of 3 independent experiments.

The predominant function of protein S-glutathionylation is to protect reactive cysteine groups from irreversible oxidation. In order to examine whether there is a functional consequence of increased protein S-glutathionylation in GSTP1-silenced HCT116 cells, cells were incubated with the alkylating agent, N-ethylmaleimide (NEM) over a 72 hour cytotoxic screen. If protein S-glutathionylation contributes to the protection of cysteine groups, then GSTP1 silenced cells should be more resistant to the thiol blocking effects of NEM. Indeed, GSTP1-silenced cells treated with NEM show increased resistance towards NEM as demonstrated in Figure 4.9, highlighting a potential functional response of increased protein S-glutathionylation as a consequence of silencing GSTP1. Although from these data we cannot determine if resistance is mediated specifically through increased thiol S-glutathionylation as other thiol modifications may also occur, the data show strong correlation between increased protein S-glutathionylation in GSTP1-silenced cells and resistance to NEM.

4.9 Protein S-glutathionylation in response to endogenous glutathione depletion

It is important to acknowledge that, so far, differences in protein S-glutathionylation may be masked by the activity of endogenous glutathione, especially when differences in these levels can be observed upon silencing of GSTP1 (Figure 4.7). It is also difficult to assess whether the increase in BioGEE incorporation is due a stress response and is a true reflection of increased protein S- glutathionylation in GSTP1 silenced cells, or the fact that more reactive thiol groups are available in GSTP1 silenced cells for BioGEE to bind. If the latter was true, it would be difficult to assess whether or not protein S-glutathionylation was increased or decreased. To circumvent this potential problem, endogenous glutathione levels were depleted using buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting step in glutathione synthesis.

Depletion of glutathione was carried out as described by Higgins et al 2009, with modifications (Higgins et al., 2009). HCT116 cells were silenced for GSTP1 as described previously. Cells were grown for 48 hours before incubated with BSO (50µM) for 6 hours. Cell culture media was then replaced with fresh media containing BSO (50µM) and incubated for a further 12 hours.



IC50 (95% confidence intervals)	
Control siRNA	GSTP1 siRNA
29.36 (27.98, 30.81)	44.2 (30.84, 63.33)

Figure 4.9. IC_{50} curve of N-ethylmaleimide treated HCT116 cells silenced for GSTP1.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA (n=4). Cells were trypinised and seeded into a 96 well plate 72 hours post transfection and incubated with different concentrations of N-ethylmaleimide (NEM). Cell viability was determined using the ATP assay 72 hours after NEM treatment. The IC50 values are shown in the table above. Values are represented as mean ± standard deviation.

Cells were treated with BioGEE (0.4mM) for 1 hour and lysates were prepared and resolved on a non-reducing SDS-PAGE gel. Western blot analysis using a streptavidin-HRP antibody demonstrates that depletion of endogenous glutathione levels enhances the sensitivity of BioGEE labelling, as increased protein labelling can be detected in control siRNA and GSTP1 siRNA treated cells after incubation with BSO (Figure 4.10A)**.** BSO (50µM) treatment reduced glutathione levels to 18% and 22% in control and GSTP1 siRNA treated HCT116 cells respectively (data not shown). In the absence of BSO, BioGEE is only able to accurately label high molecular weight proteins as smaller proteins are already saturated by endogenous glutathione. After endogenous glutathione depletion, BioGEE is more readily incorporated into smaller molecular weight proteins and therefore increases the resolution of proteins S-glutathionylated. BioGEE labelling is greatly enhanced in GSTP1-silenced cells, demonstrating increased protein S-glutathionylation after endogenous glutathione depletion when compared to control cells. This may be due to an increase in the reactivity of the cysteine groups after silencing of GSTP1, evident from the observation that levels of ROS increases after BSO treatment in GSTP1-silenced HCT116 cells as determined by H_2DCFDA staining (Figure 4.11). However, a lack of HO-1 induction suggests that the level of oxidation in the cell does not warrant induction of other stress response pathways. These data demonstrate that GSTP1 is not required for the S-glutathionylation of cellular proteins in HCT116 cells as proteins are more readily S-glutathionylated in the absence of GSTP1.

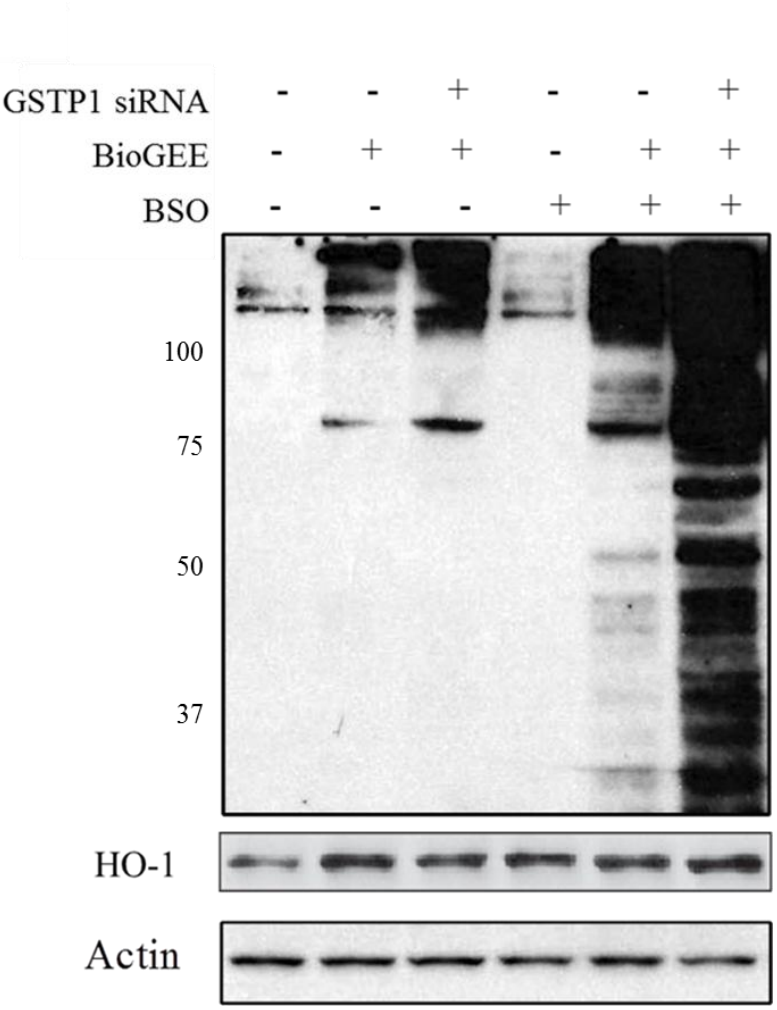


Figure 4.10. Increased protein S-glutathionylation in GSTP1-silenced HCT116 cells after endogenous glutathione depletion.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA (n=3). Cells were grown for 48 hours before incubated with BSO (50µM) for 6 hours. Cell culture media was then replaced with fresh media containing BSO (50µM) and incubated for a further 12 hours before treated with BioGEE (0.4mM) for 1 hour. Cells were harvested and lysates (10µg) were resolved on a non-reducing SDS-PAGE gel and analysed by Western blotting using a streptavidin-HRP antibody (1:1000).

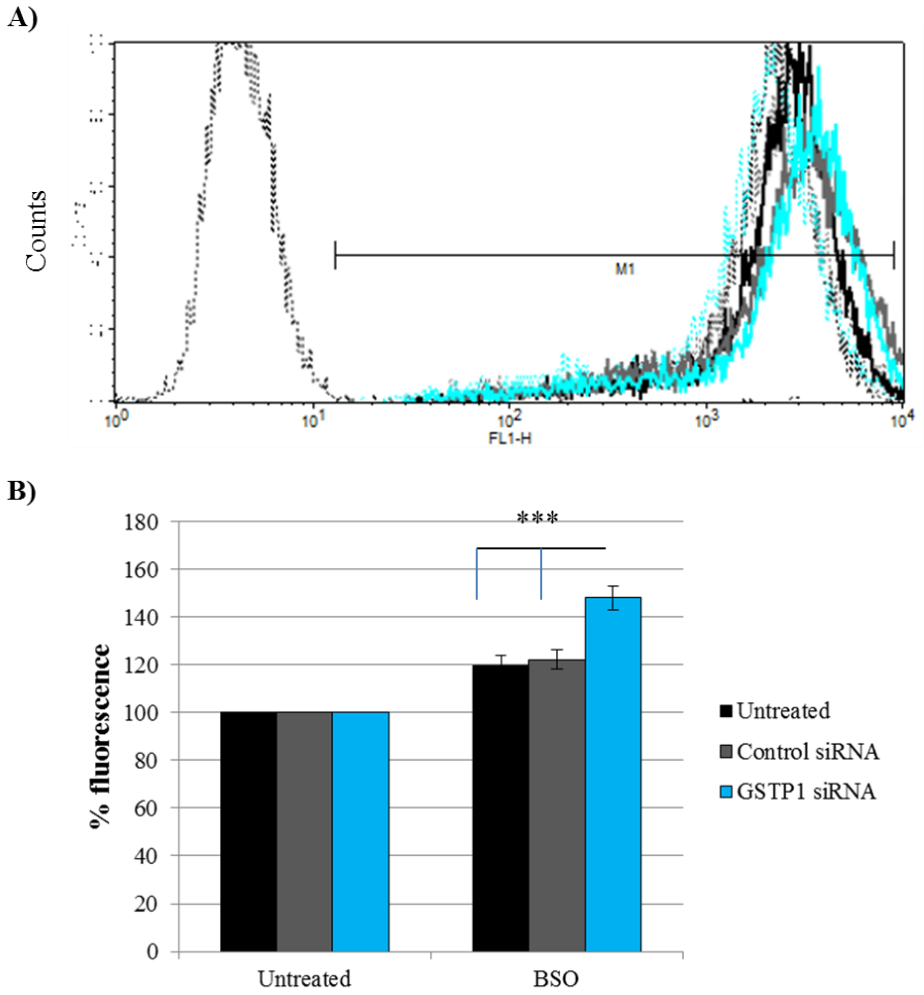


Figure 4.11. ROS activity after depletion of endogenous glutathione levels.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA (n=3). Cells were grown for 48 hours before incubated with BSO (50µM) for 6 hours. Cell culture media was then replaced with fresh media containing BSO (50µM) and left for 12 hours. Cells were incubated with H_2DCFDA (10µM) in PBS for 30 minutes before trypsinised, washed in PBS and analysed by flow cytometry. **A)** Histogram plot of HCT116 cells treated with H_2DCFDA. The left hand peak is cells treated with PBS only and used as a negative control against H_2DCFDA staining. **B)** The bar chart represents the area under each histogram plot. Fluorescence is shown as a percentage of untreated HCT116 cells. Values are represented as mean ± standard deviation, where *** $P < 0.001$.

4.10 Effect of NOS signalling on protein S-glutathionylation

As discussed previously, there are a number of ways in which proteins can become S-glutathionylated. In the context of silencing GSTP1 in HCT116 cells, a rise in glutathione disulphide content (Figure 4.7B) as a result of increased disulphide exchange could account for the increase in protein S-glutathionylation observed in GSTP1 silenced HCT116 cells (Figure 4.8). However, it could also be hypothesised that an increase in protein nitrosylation or formation of nitrosyl- intermediates may act as a precursor to protein S-glutathionylation. In order to examine this hypothesis, HCT116 cells silenced for GSTP1 were incubated with N-Nitro-L-arginine methyl ester hydrochloride (L-NAME), an analogue of arginine that inhibits nitric oxide (NO) production. Cells were silenced for GSTP1 using siRNA as previously described and then incubated with 5, 50 or 500μM L-NAME for 7 hours. BioGEE (0.4mM) was added for 1 hour after L-NAME treatment and the level of protein S-glutathionylation determined from Western blot analysis.

Pre-treatment of HCT116 cells with L-NAME does not appear to greatly reduce the expression of protein S-glutathionylation in HCT116 cells, even at high concentrations of L-NAME (Figure 4.12). The data presents a crude analysis of NO contribution to protein S-glutathionylation. However, in principle it suggests that NO production does not contribute greatly to increased protein S-glutathionylation in GSTP1 silenced HCT116 cells. Our data therefore suggest that increased disulphide exchange between reduced and oxidised glutathione may account for the increased protein S-glutathionylation. However, if this mechanism is due to slight variations in the oxidation state of the cell upon silencing GSTP1, it is difficult to conceive why an increase in the oxidation of glutathione does not correlate with increases in other redox mechanisms such as nitrosylation. Therefore, although the current data imply increased disulphide exchange as a mechanism of increased protein S-

glutathionylation in GSTP1 silenced HCT116 cells, other cellular processes cannot be fully excluded.

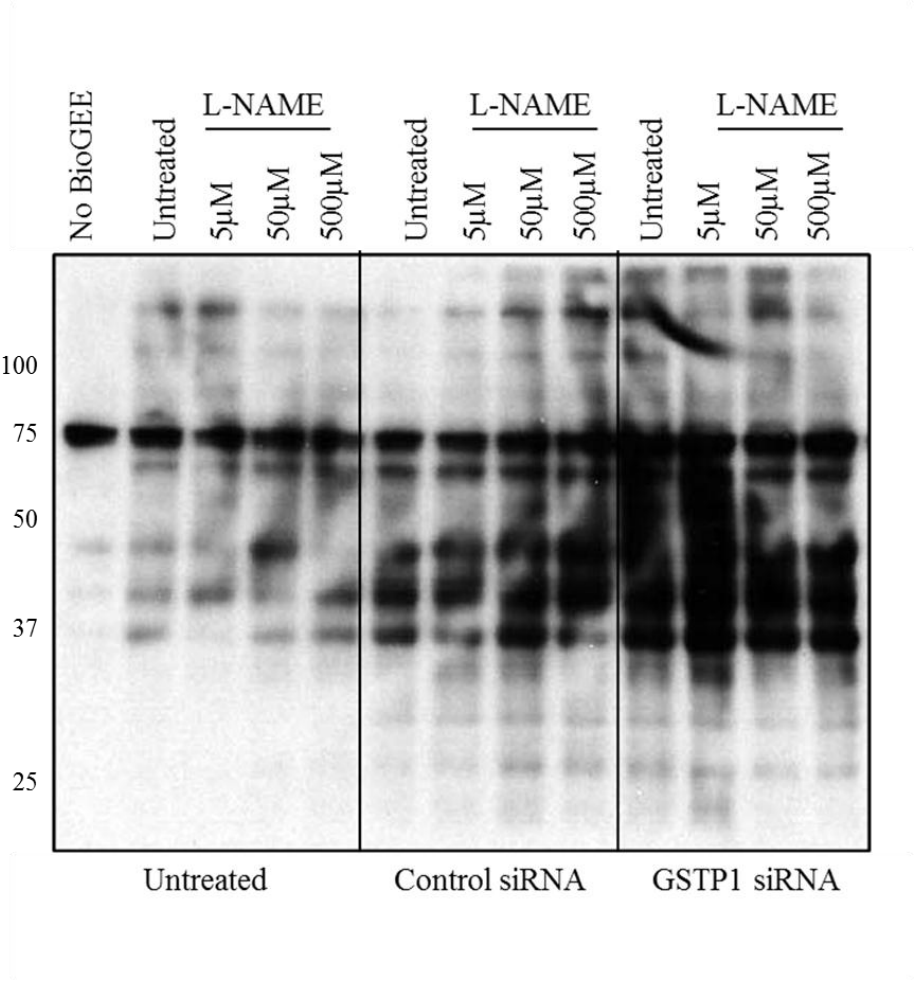


Figure 4.12. Inhibition of NOS signalling on the activity of protein S-glutathionylation.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA and grown for 72 hours. Cells were treated with different concentrations of the nitric oxide synthase inhibitor, L-NAME, for 7 hours before incubated with BioGEE (0.4mM) for 1 hour. Cells were harvested and lysates (10µg) were resolved on a non-reducing SDS-PAGE gel and analysed by Western blotting using a streptavidin-HRP antibody (1:1000). Blots are representative of 3 independent experiments.

4.11 Increase in the protein S-glutathionylation of mitochondrial proteins

So far the data have examined the expression of global protein S-glutathionylation from whole cell lysate. To enhance the identification and localisation of proteins which are S-glutathionylated, fractionation of subcellular compartments was performed in GSTP1 silenced HCT116 cells after 1 hour incubation with BioGEE (0.4mM) by centrifugal differentiation. Cytosolic and mitochondrial lysates (10µg) were resolved on a non-reducing SDS-PAGE gel and analysed by Western blotting (Figure 4.14). Lactate dehydrogenase (LDH) is localised within the cytosol and its expression in Figure 4.14 shows that there is little contamination of mitochondrial preparations from cytosolic fractions. Manganese superoxide dismutase (MnSOD) is predominantly localised to the mitochondria and in this study, demonstrates neat quantification of mitochondria extracted from HCT116 cells. From Western blot analysis, we can clearly identify a number of mitochondrial proteins which are S-glutathionylated and can observe an increase in protein S-glutathionylation in HCT116 cells silenced for GSTP1. However, we do not observe a large proportion of protein S-glutathionylation from cytosolic preparations, which is of some concern as the majority of S-glutathionylated proteins to date have predominantly localised in the cytosol. The nature of the subcellular fractionation procedure, which results in heavily diluted cytosolic fractions, may account for this observation. The apparent increased protein S-glutathionylation within the mitochondria is of great interest in relation to GSTP1-mediated cytoprotection, where the localisation and function of GSTP1 within the mitochondria was previously discussed and analysed in Results Chapter 3. As mentioned previously, mitochondria cannot export oxidised glutathione. In times of oxidative stress, S-glutathionylation of mitochondrial proteins reduces the level of oxidised glutathione in the mitochondria in order to reduce mitochondrial damage as a result of increased ROS. Silencing of GSTP1 in HCT116 cells does not appear to be detrimental to the integrity of the mitochondria as was evident

examining various apoptotic parameters by flow cytometry in Figure 4.4B. Transmission electron microscopy (TEM) of mitochondria in HCT116 cells silenced for GSTP1 supports this observation, as very few changes can be identified in the mitochondrial structure upon silencing of GSTP1 (Figure 4.13). A possible mechanism is that in the absence of GSTP1, mitochondrial oxidative stress increases, which increases protein S-glutathionylation as a way of attempting to maintain redox homeostasis by reducing the concentration of GSSG. The results demonstrate a novel potential function of GSTP1 in relation to mitochondrial regulation. The role in GSTP1-mediated cytoprotection of the mitochondria is analysed and discussed later in the chapter in relation to protein-specific regulation.

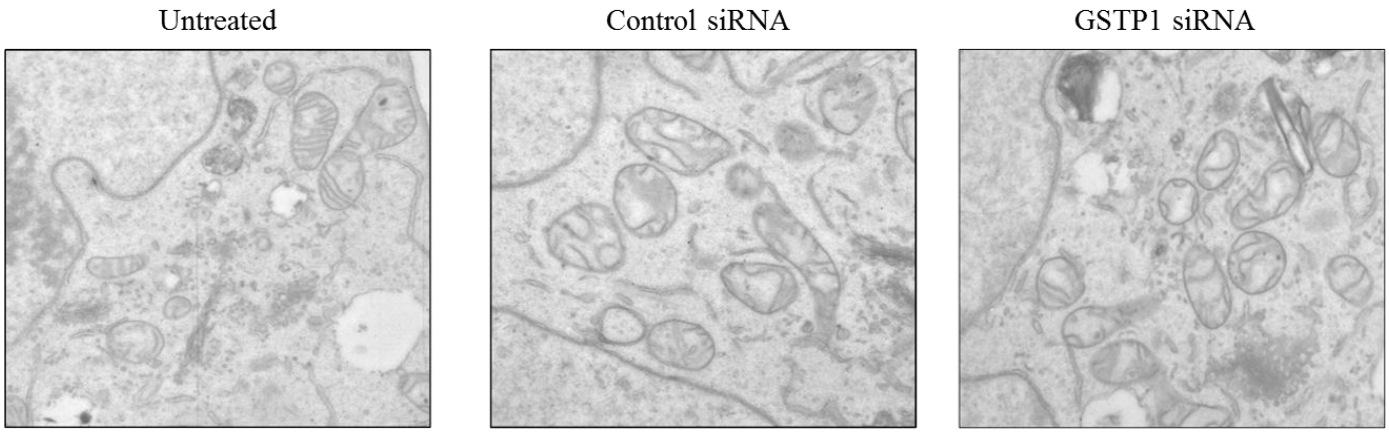


Figure 4.13. Analysis of mitochondria in GSTP1 silenced HCT116 cells using transmission electron microscopy.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA and grown for 72 hours. Cells were fixed in2% PFA in 0.1M Cacodylate buffer and post fixed in 1% aqueous osmium. Cells were dehydrated in graded alcohols and incubated in propylene oxide before placed in neat resin and analysed by transmission electron microscopy. Pictures are representative of 3 independent experiments. All images are at a magnification of 10,000x.

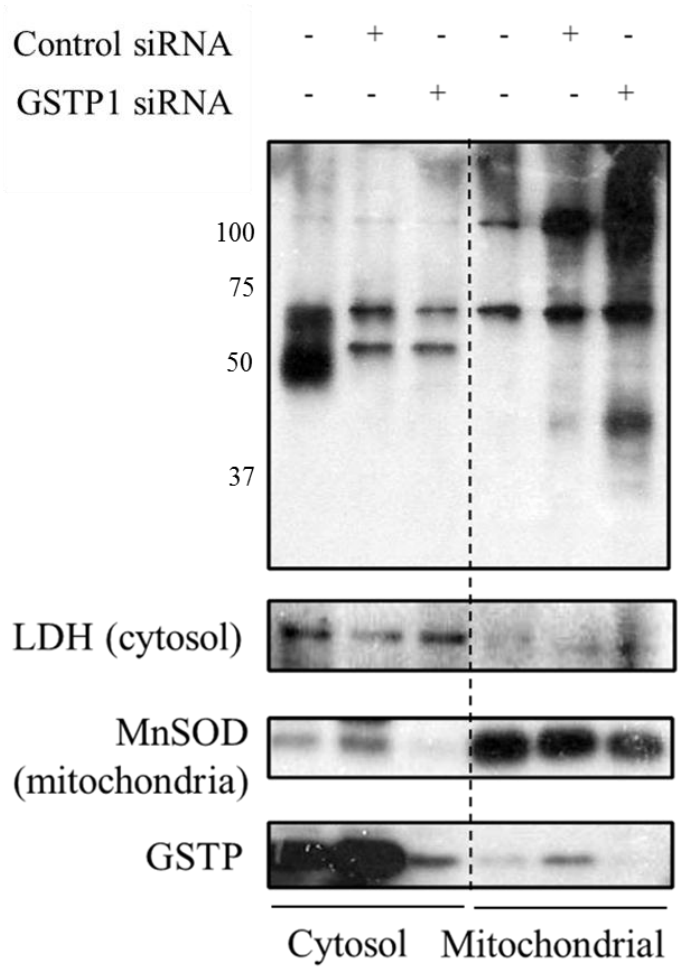


Figure 4.14. Analysis of protein S-glutathionylation of cytosolic and mitochondrial fractions in GSTP1 silenced HCT116 cells.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA and grown for 72 hours. Cells were incubated with BioGEE (0.4mM) for 1 hour before cytosolic and mitochondrial fractions were prepared by differential centrifugation as described in 'Materials and Methods'. Lysates (10µg) were resolved on a non-reducing SDS-PAGE gel and analysed by Western blotting using antibodies against LDH (cytosolic marker, 1:1000), MnSOD (mitochondrial marker, 1:1000), GSTP1 (1:2000) and a streptavidin-HRP antibody (1:1000). Blots are representative of 3 independent experiments.

4.12 Two-dimensional gel electrophoresis analysis of protein S-glutathionylation in GSTP1 silenced HCT116 cells

Further to analysing protein S-glutathionylation through subcellular fractionation, individual S-glutathionylated proteins were separated and analysed by two-dimensional gel electrophoresis (2DE). GSTP1 was transiently silenced in HCT116 cells as described previously. Cells were incubated with BioGEE (0.4mM) for 1 hour and harvested in a non-reducing lysis buffer. Proteins (100µg) were acetonitrile precipitated and resuspended in isoelectric focusing (IEF) buffer (100µl). Proteins were subsequently separated on Immobilised pH Gradient (IPG) ReadyStrips™ pH3-10 NL using a Protean IEF Cell (BioRad) as described in 'Materials and Methods'. Western blot analysis of biotinylated proteins was performed using a streptavidin-HRP antibody, while silver staining of 2DE gels was performed to analyse sample loading accuracy. Analysis of the protein expression profiles highlights a number of individual proteins which appear to be S-glutathionylated (Figure 4.15). Similarly to 1 dimensional gel electrophoresis, the majority of proteins identified in GSTP1 silenced HCT116 cells show increased S-glutathionylation compared to control siRNA treated cells. However, there does appear to be a cohort of proteins in which the biotinylation signal is markedly reduced or absent, highlighted in blue, implying that there may be a subset of proteins in which their ability to become S-glutathionylated is actively catalysed by GSTP1. Initial 2DE experiments have proven difficult to accurately identify individual proteins using mass spectrometric analysis from a crude lysate due to the cleanliness of the streptavidin-HRP antibody and an optimised approach for the identification of S-glutathionylated proteins is detailed in the 'Discussion' Chapter.

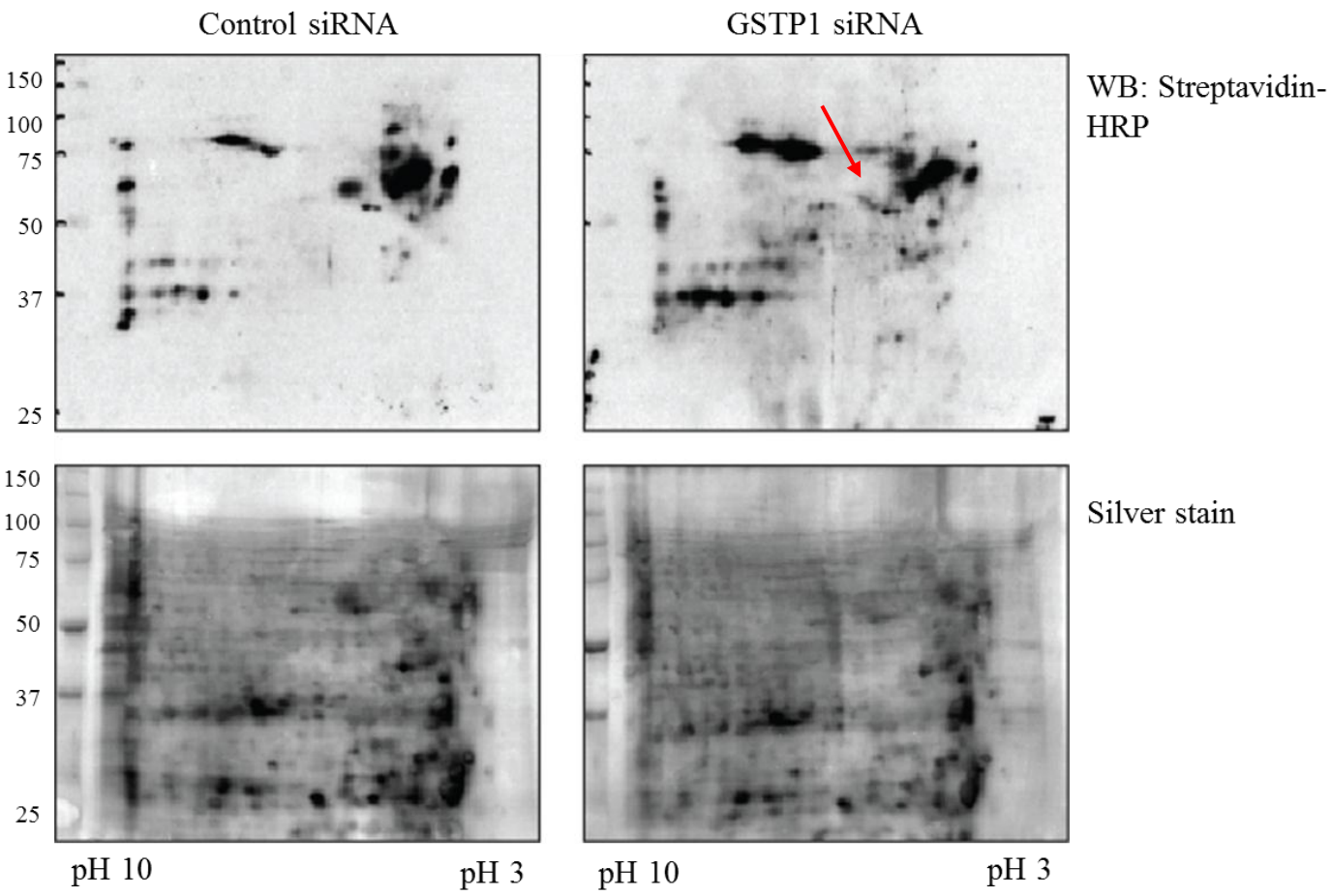


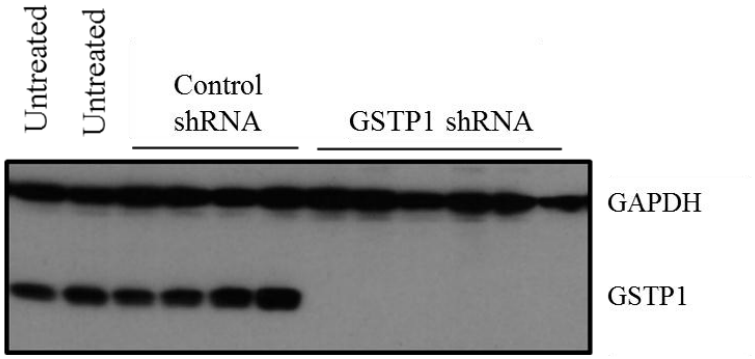
Figure 4.15. Analysis of protein S-glutathionylation in GSTP1 silenced HCT116 cells by two-dimensional gel electrophoresis.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA and grown for 72 hours. Cells were incubated with BioGEE (0.4mM) for 1 hour and harvested in non-reducing lysis buffer. Proteins were acetonitrile precipitated (100µg) and resuspended in 100µl of isoelectric focusing (IEF) buffer. Proteins were separated using two-dimensional gel electrophoresis and protein S-glutathionylation was analysed by Western blot analysis using streptavidin-HRP while equal loading was determined by silver stain (70µg protein for Western blot, 20µg protein for silver stain). The red arrow highlights a potential group of proteins which may be deglutathionylated upon silencing of GSTP1. Blots are representative of 3 independent experiments.

4.13 Stable knockdown of GSTP1 in HCT116 cells using shRNA

The study has utilised siRNA to target GSTP1 knockdown in HCT116 cells, but this approach becomes limited when examining the long term effects of silencing GSTP1. Therefore, by creating a stably silenced GSTP1 HCT116 cell line, the accurate nature of protein S-glutathionylation mediated by GSTP1 can be determined. GSTP1 was stably silenced in HCT116 cells using lentiviral transduction of plasmids expressing short hairpin RNA (shRNA) constructs targeted against GSTP1 or expressing no shRNA (plasmid/negative control). Several shRNA constructs targeted against different regions of *GSTP1* mRNA transcript were examined in HCT116 cells, of which only one shRNA construct produced a knockdown of over 60% as determined by rtPCR (Taqman) (data not shown) and was subsequently used for stable knockdown of GSTP1. After lentiviral transduction, positive clones were identified under puromycin selection. After colony selection and clonal expansion, cells were examined for GSTP1 protein expression by western blot analysis and transcript analysis using rtPCR. shRNA expression targeting GSTP transcript was driven from a pol III U6 promoter cloned into the pLKO.1 vector. Expression of shRNA from this promoter results in stable knockdown of GSTP1 in HCT116 cells as is evident from both protein expression analysis (Figure 4.16A) and transcript analysis (Figure 4.16B). Expression of the plasmid only, used as a negative control, has little effect on GSTP1 protein levels, whilst some clones showed slight differences in *GSTP1* mRNA expression. The same clones were used for all ensuing studies but where more than one clone is used, this is highlighted in the text and figures.

A)



B)

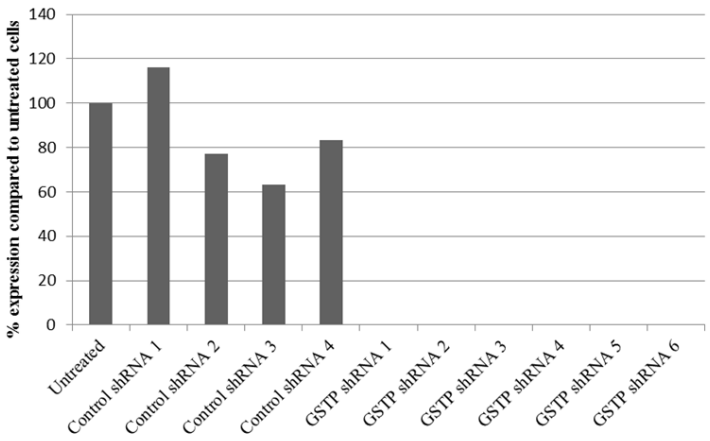


Figure 4.16. Analysis of GSTP1 expression after lentiviral transduction of shRNA targeting GSTP1 in HCT116 cells.

HCT116 cells were lentivirally transduced with shRNA plasmids targeting GSTP1 or an empty plasmid (control). After colony selection and clonal expansion, cells were examined for GSTP1 protein expression by western blot analysis **(A)** and transcript analysis using rtPCR (Taqman) **(B).** For Western blot analysis, cell lysates (10µg) were resolved on a SDS-PAGE gel and analysed using antibodies targeting GSTP1 (1:2000) whilst sample loading was analysed using an antibody targeting GAPDH (1:5000). For rtPCR, 1µg RNA was synthesised to cDNA, diluted 1:80 and used in a duplex Taqman reaction using probes targeting GSTP1 and 18S as an endogenous loading control. ΔΔCt values were determined from the analysis and are displayed as percentage of expression in comparison to untreated HCT116 cells.

4.14 Effect of stable knockdown of GSTP1 on the proliferation of HCT116 cells

Transient silencing of GSTP1 in HCT116 cells results in reduced proliferation of HCT116 cells, possibly due to reduced progression through mitosis as is evident from changes in cell cycle profiles (Figure 4.4A). Similarly to transient silencing of GSTP1, stable knockdown of GSTP1 cells significantly increases the doubling time of a number of GSTP1 knockdown HCT116 clones ($HCT116^{shGSTP}$) over a 72 hour period (Table 9). Therefore, GSTP1 appears to have an intrinsic function in the proliferation of HCT116 cells. This is in line with previous studies demonstrating deletion of GSTP1 in HCT116 cells results in reduced cell growth (Dang et al., 2005). In order to determine whether this was a result of changes in cell cycle parameters, the cell cycle profile of individual $HCT116^{shGSTP1}$ clones were analysed by flow cytometry (Figure 4.17). Unlike transient silencing of GSTP1 using siRNA, stable knockdown does not induce changes of cell cycle parameters in HCT116 cells, indicating that another mechanism may account for the reduced proliferation and not necessarily slower progression though mitosis.

Genotype	Doubling time (hrs)
Untreated	23.43 ± 1.17
Control shRNA	23.13 ± 4.8
GSTP1 shRNA	31.96 ± 4.5*

Table 9. Doubling time (hrs) of HCT116 cells after stable knockdown of GSTP1.

HCT116 cells were transduced with lentiviral plasmids expression shRNA targeting GSTP1 ($HCT116^{shGSTP1}$) or no shRNA ($HCT116^{shcontrol}$). Several individual clones isolated from the initial puromycin selection screen were used in this experiment (untreated HCT116 cells, n=3; control shRNA , n=4; GSTP1 shRNA, n=6). Cells were seeded onto a 96 well plate at 1500 cells per well and the doubling time was assessed 72 hours later using the ATP assay. Values are represented as mean ± standard deviation, where * GSTP1 vs control shRNA $P < 0.05$.

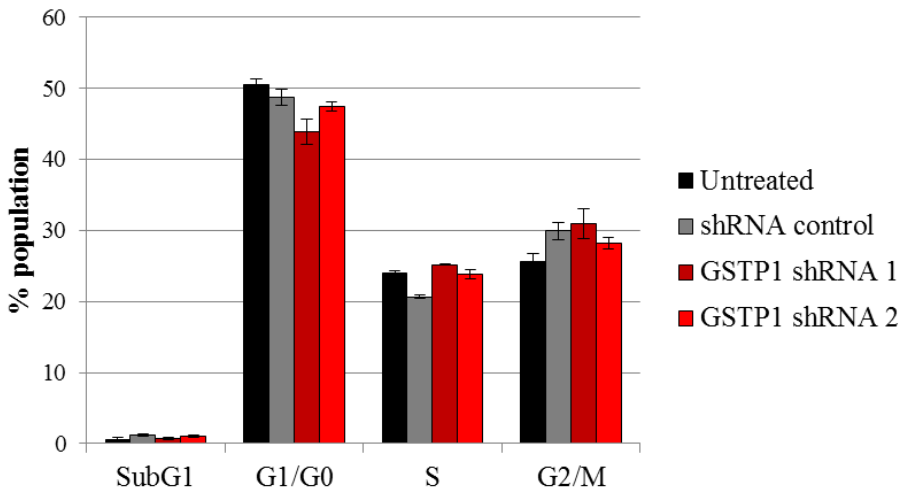


Figure 4.17. Cell cycle profile analysis of stable knockdown of GSTP1 in HCT116 cells.

The cell cycle profile of individual $HCT116^{shGSTP1}$ clones were analysed by flow cytometry. Prior to analysis, cells were seeded onto 6 well plates and grown in puromycin free media to 70% confluency. Cells were washed in PBS and fixed in 70% ethanol before stained with propidium iodide. Cell cycle profiles were analysed by flow cytometry. Values are represented as mean ± standard deviation.

4.15 Analysis of reduced and oxidised glutathione content after stable knockdown of GSTP1 in HCT116 cells

Transient silencing of GSTP1 in HCT116 cells results in increases on reduced and disulphide glutathione levels (Figure 4.7). These parameters were also examined in stable HCT116 cell lines silenced for GSTP1. In contrast to transient knockdown of GSTP1, no difference in total glutathione concentrations could be determined in HCT116^{shGSTP1} cells (Figure 4.18A). However, there appears to be a marked increase in the proportion of oxidised glutathione in 3 individual HCT116^{shGSTP1} clones (Figure 4.18B), suggesting that knockdown of GSTP1 induces changes in the oxidation state of the cell. These data are comparable to that of transient silencing of GSTP1 in HCT116 cells which suggests a uniform mechanism in GSTP1-mediated cytoprotection in HCT116 cells.

4.16 Protein S-glutathionylation in stably silenced GSTP1 HCT116 cells

In relation to protein S-glutathionylation, the results generated from transient knockdown of GSTP1 led to the hypothesis that increased oxidation of glutathione may lead to increased disulphide exchange and, as a consequence, may have led to increased protein S-glutathionylation. As a result of increased oxidation of glutathione in stably silenced HCT116 cells, the level of protein S-glutathionylation was examined to test the disulphide hypothesis and examine the effect of GSTP1-mediated S-glutathionylation. HCT116 cells were treated with BSO as described previously, to maximise the resolution of proteins which are S-glutathionylated. Upon treatment with BioGEE (0.4mM) there does not appear to be any difference in the level of protein S-glutathionylation between HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells treated with BSO as determined by Western blot analysis (Input, Figure 4.19). Cells were also treated with the thiol oxidant, diamide, for 15 minutes after BioGEE incubation to induce protein S-glutathionylation and therefore determine if GSTP1-mediated

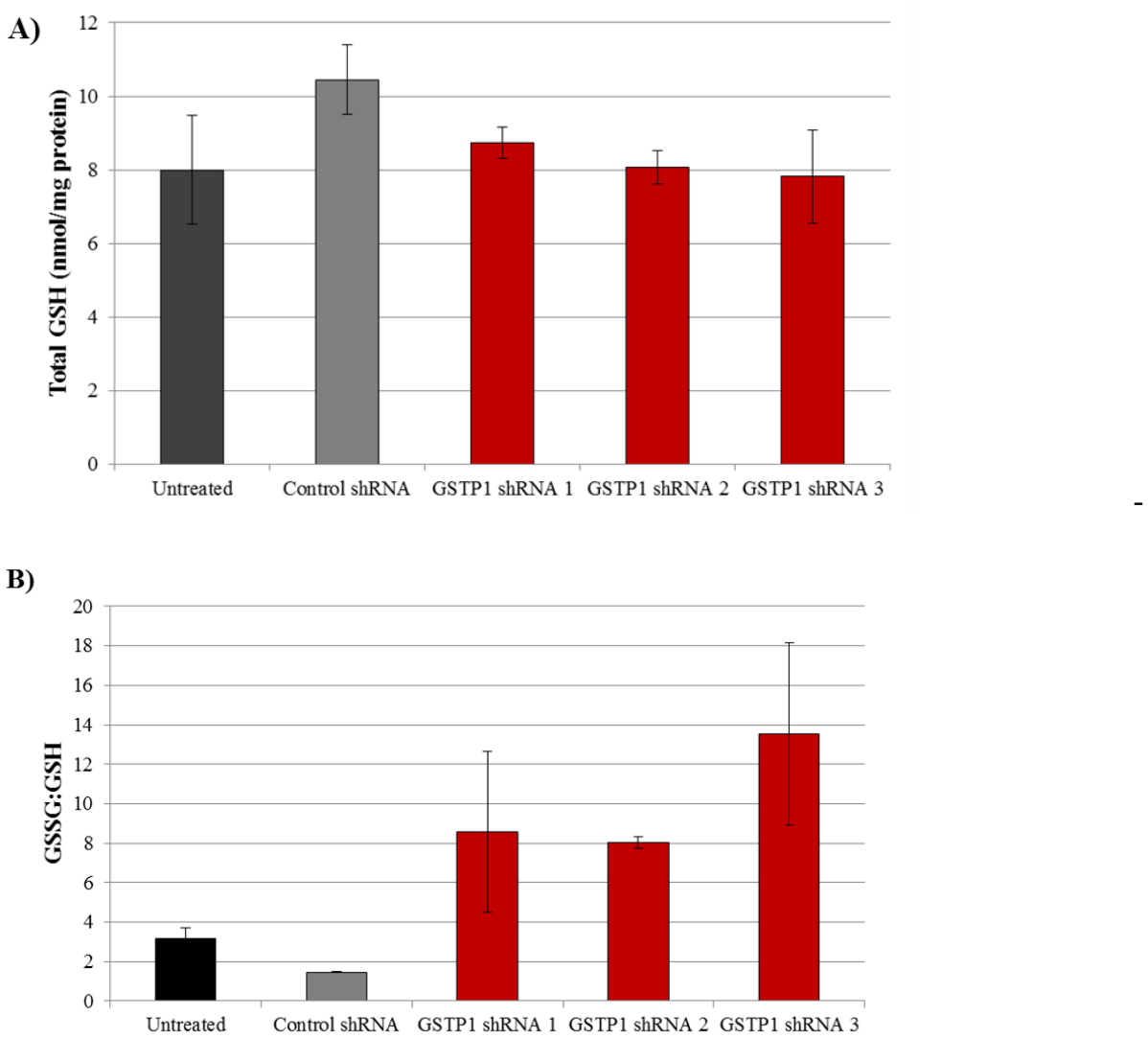


Figure 4.18. Analysis of total and oxidised glutathione levels in HCT116 cells stably silenced for GSTP1.

HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells were analysed for total **(A)** and oxidised **(B)** levels of glutathione (n=3). For HCT116^{shGSTP1} cells, 3 individual clones were analysed in the assay. Values are represented as mean ± standard deviation.

S-glutathionylation was stress dependent. There was no apparent difference in the level of protein S-glutathionylation after diamide treatment across all cell lines. Biotinylated proteins were immunoprecipitated from cell lysates using streptavidin coupled Dynabeads, to determine any subtle differences in S-glutathionylated proteins. Cell lysates (500mg) were incubated with streptavidin-coated Dynabeads® (40µl) at 4°C for 1 hour under gentle rotation before biotinylated proteins were separated from streptavidin beads by heating the samples at 95°C for 5 min in a non-reducing sample buffer. Immunoprecipitation of biotinylated proteins from BSO and diamide treated cells demonstrates a difference in the composition of proteins S-glutathionylated after BSO and diamide treatment, with BSO enhancing the resolution of S-glutathionylated proteins to a greater extent than diamide. However, there is no apparent difference in the extent of biotinylation when compared to HCT116^{shGSTP1} cells (IP, Figure 4.19). The results demonstrate that stable knockdown of GSTP1 does not lead to decreased protein S-glutathionylation in HCT116 cells, even in the presence of the oxidant diamide, indicating that it is unlikely to catalyse protein S-glutathionylation in this context. However, the results do not resemble those observed in transiently silenced HCT116 cells, which may allude to the transient function of this modification or highlight compensatory mechanisms which may account for the stable loss of GSTP1.

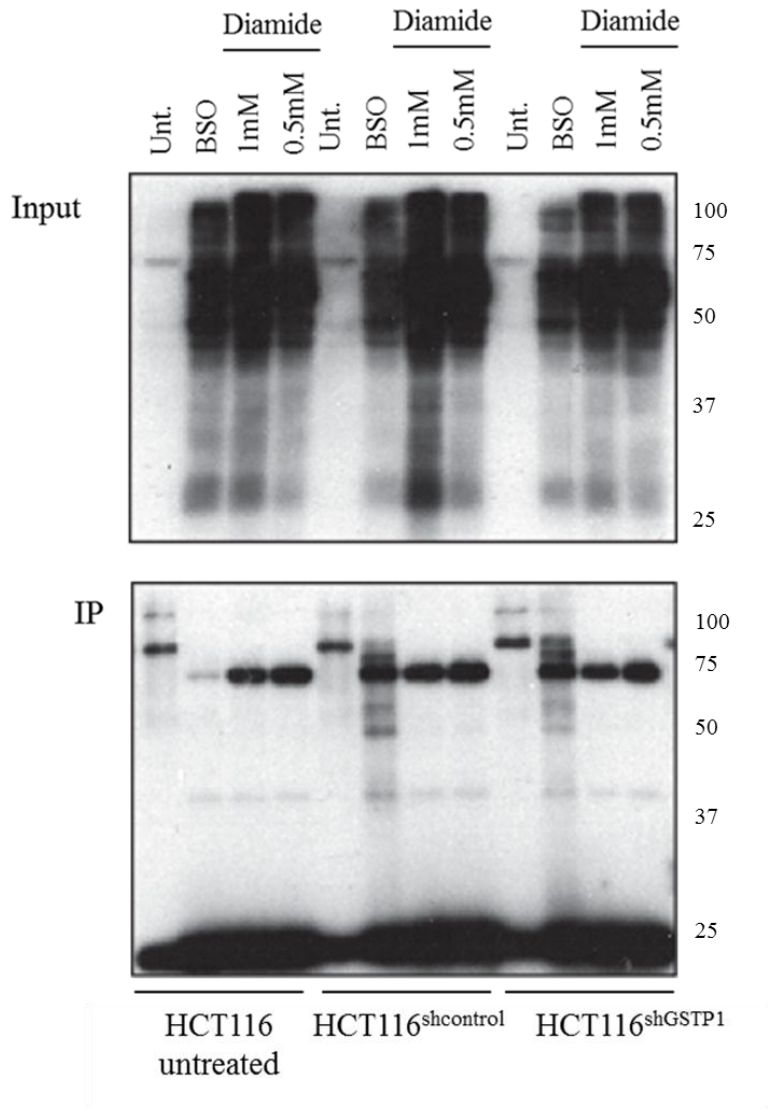
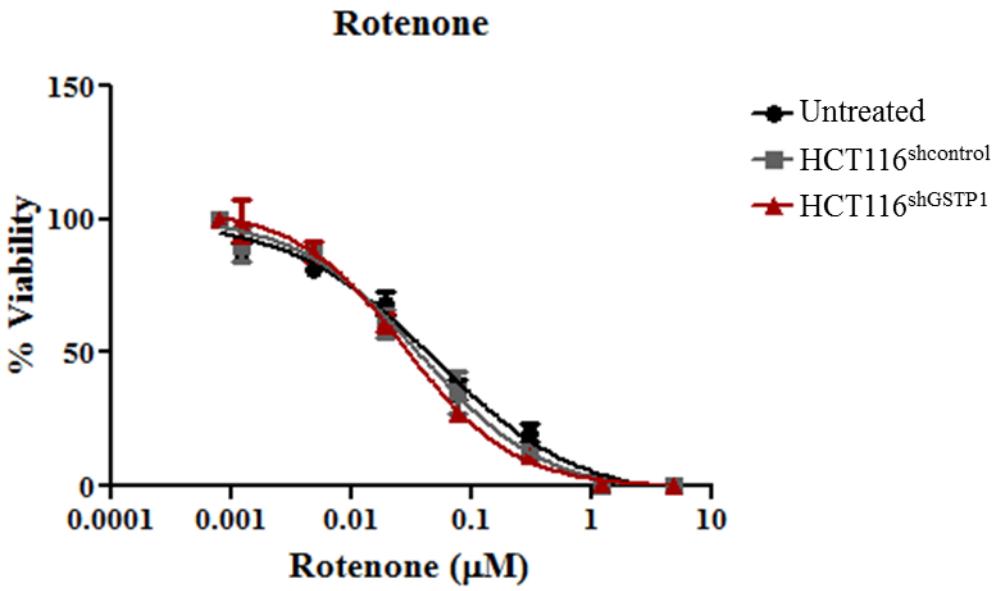


Figure 4.19. Protein S-glutathionylation in HCT116 cells stably silenced for GSTP1.

HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells were incubated with BSO (50µM) for 6 hours. Cell culture media was then replaced with fresh media containing BSO (50µM) and incubated for a further 12 hours before treated with 0.4mM BioGEE for 1 hour. As an alternative to BSO, protein S-glutathionylation was induced in cells after treatment with diamide for 15 minutes post BioGEE incubation. Lysates were prepared and immunoprecipitated (500mg) using streptavidin-coupled Dynabeads® for 1 hour at 4°C. Proteins were separated from beads by boiling in 50µl non-reducing LDS buffer before 10µl was resolved on a SDS-PAGE gel. Gels were analysed by Western blotting using a streptavidin-HRP antibody (1:2000). Blots are representative of 3 independent experiments.

4.17 GSTP1 regulation of mitochondrial proteins

Transient silencing of GSTP1 in HCT116 cells resulted in increased S-glutathionylation of mitochondrial subcellular fractions but did not induce changes in mitochondrial integrity or structure (Figure 4.13). In HCT116 cells treated with the mitochondrial toxicant, rotenone, stable silencing of GSTP1 demonstrates a slight increase in cell sensitivity over a 72 hour cytotoxicity study (Figure 4.20). Although not significant there is a trend which suggests GSTP1 may play a role in the protection of mitochondria against rotenone treatment. However, it must be acknowledged that slight differences may, in part, be due to decreased cell numbers during the course of rotenone treatment due to decreased cell proliferation in HCT116^{shGSTP1} cells (Table 9). To examine the role of GSTP1 in the mitochondria in more detail, levels of oxygen consumption and glycolysis were determined in response to mitochondrial stress using the XF24 Analyser (Seahorse Biosciences). HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells were seeded at 20,000 cells per well onto a 24 well plate and incubated overnight. The following morning, cell media was replaced with unbuffered culture media and cells were incubated for 1 hour at 37°C in the absence of carbon dioxide. The rate of glycolysis and oxygen consumption was measured in response to DNP treatment. After 5 basal readings were taken, cells were treated with DNP (100µM) and the rate of oxygen consumption (OCR) and glycolysis (ECAR) measured. There was no difference in basal OCR and ECAR in two individual HCT116^{shGSTP1} cell lines compared to control cells (Figure 4.21), suggesting that GSTP1 is not required for basal mitochondrial function in HCT116 cells. After treatment with DNP, OCR and ECAR rates increased across all cell lines. However, there is no significant difference in the rate of OCR and ECAR in the absence of GSTP1, suggesting that in HCT116 cells, mitochondrial function does not appear to be intrinsically regulated by GSTP1.



IC50 (95% confidence intervals)		
Untreated	HCT116^{shcontrol}	HCT116^{shGSTP1}
0.051 (0.036, 0.071)	0.039 (0.029, 0.052)	0..30 (0.023, 0.034)

Figure 4.20. Cytotoxicity assay examining the effect of the mitochondrial toxicant, rotenone, in the absence of GSTP1 in HCT116 cells.

HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells were seeded into a 96 well plate at 1500 cells/well and treated with rotenone 24 hours later (n=4). After 72 hours incubation, cell viability was determined using the ATP assay. Values are represented as mean ± standard deviation.

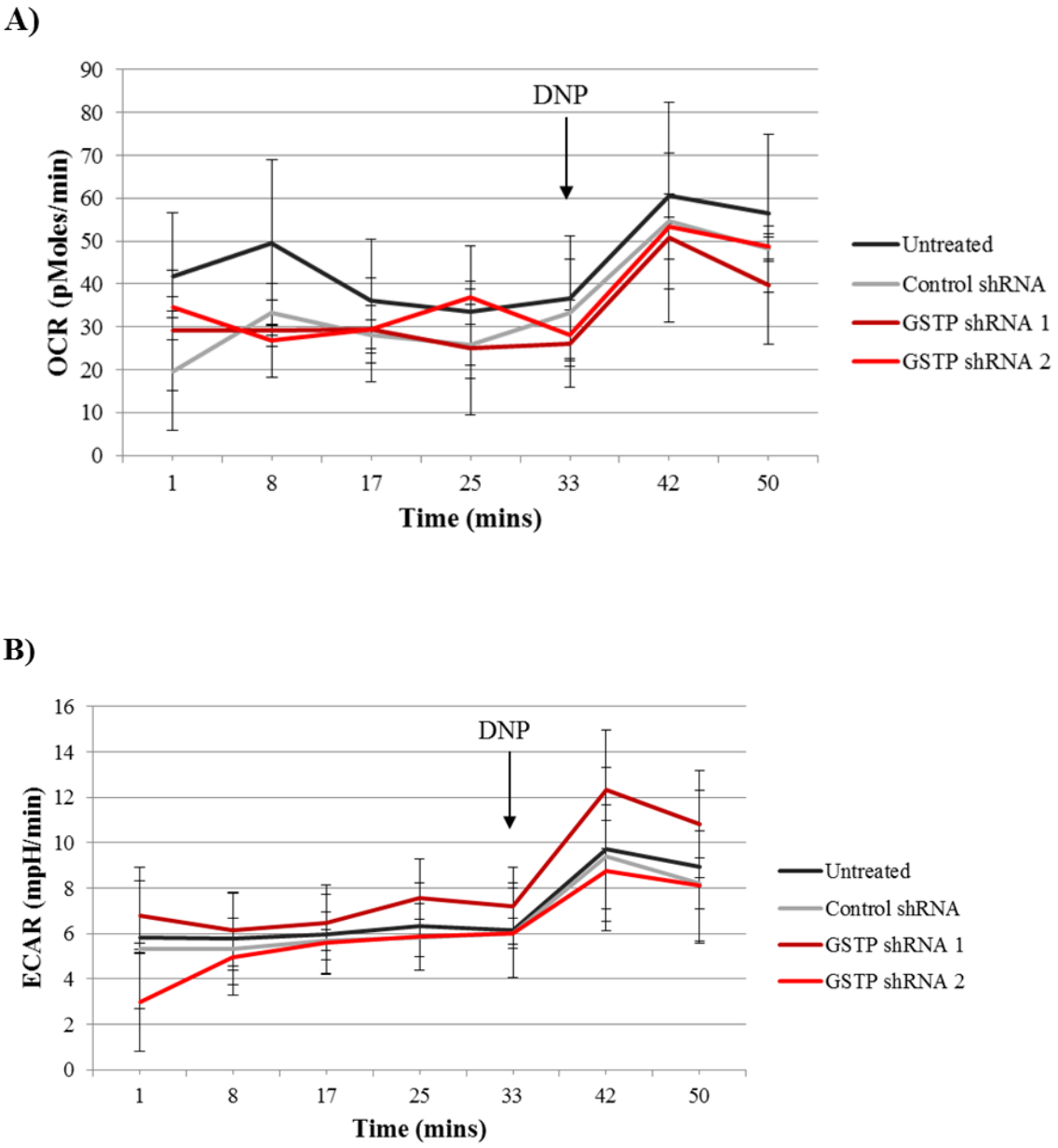


Figure 4.21. Mitochondrial respiratory function of GSTP1 in response to 2,4-dintrophenol (DNP).

Oxygen consumption rates **(A)** and levels of glycolysis **(B)** were analysed in HCT116, HCT116 ^{shcontrol} and 2 individual HCT116^{shGSTP1} cell lines using the XF24 Analyser (n=3). Cells were incubated in unbuffered media for 1 hour prior to analysis. After 5 initial readings, DNP (100µM) was injected onto cells. Data show mean ± standard deviation.

However, a number of mitochondrial proteins have been identified as potential interacting partners with GSTP1 and therefore the role of GSTP1 could potentially be in the regulation of these proteins. Unpublished data from a Yeast two hybrid screen demonstrates GSTP1 can associate with a number of mitochondrial proteins (Table 10). Of particular interest is the strong association between GSTP1 and the mitochondrial protein electron transfer flavoprotein (ETFα). ETFα is an electron acceptor for a number of dehydrogenases and participates in catalysing the initial step of the mitochondrial fatty acid ß-oxidation (Furuta et al., 1981). To examine possible interactions between the two proteins, GSTP1 cloned from HCT116 cDNA was N-terminally tagged with GFP and transfected into HCT116 cells stably silenced for GSTP1. The GSTP1-GFP constructs contain silent mutations within the shRNA binding sequence, preventing silencing by shRNA whilst keeping the amino acid composition in the *GSTP1* open reading frame intact. By performing the experiment as such, GFP-tagged constructs do not have to compete out endogenous GSTP1 and therefore a clean immunoprecipitation can be achieved at physiological concentrations. In addition, a non-catalytic GSTP1 construct harbouring a Y7F mutation was cloned into GFP to assess non-catalytic properties of GSTP1.

What is particularly interesting is that GSTP1 present within HCT116 cells contains a polymorphism in the *GSTP1* gene, a I105V substitution (*B polymorphism). In the context of colon carcinoma cell lines, it is not known whether this polymorphism contains any functional properties different to that of wild-type GSTP1. As described in the 'Introduction' chapter, I105V has been found to possess less catalytic activity than wild-type GSTP1 towards CDNB *in vitro*, but has more catalytic activity towards certain epoxide containing

compounds. To control for the effects of the polymorphism in HCT116 cells, a V105I substitution was cloned into HCT116 GSTP1 which reverts GSTP back into its wild-type conformation (*A), as described by its GST nomenclature. For the purpose of this study, GSTP1 constructs labelled as GSTP1*B refer to polymorphic GSTP1 which is endogenous to HCT116 cells, whereas GSTP1*A refers to wild-type GSTP1. GSTP1^{Y7F} constructs have been cloned against HCT116 GSTP1 and therefore also contain the *B polymorphism.

Number of hits	Gene
25	Electron transfer flavoprotein (ETFα)
12	Metallothionein 2
5	NADH dehydrogenase (ubiquinone) iron-sulphur protein 6
1	NADH-ubiquinone oxidoreductase chain 1
1	Ancient ubiquitous protein 1 (AUP1)
1	Plasminogen
1	Fas binding factor
1	Na+/K+ ATPase, ß subunit 1
1	Sorting nexin 3 (SNX3)
1	Novel gene
1	X-linked anhidroitic ectodermal dysplasia protein

Table 10. Yeast two hybrid screen of genes associated with human GSTP1.

A yeast 2 hybrid screen was performed using human GSTP1 as bait. Results kindly provided by Aileen McLaren.

HCT116^{shGSTP1} cells were seeded into 60mm culture dishes and grown until 70% confluent. Cells were transfected with 3µg of GFP-tagged GSTP1 using LTX PLUS as described in the 'Materials and Methods'. The amount of GFP-tagged construct to be used was determined by transfecting serial concentrations of GFP-tagged GSTP1 and comparing its expression to that of endogenous GSTP1 from HCT116 cells. The concentration of GSTP1 transfected closely resembles the physiological expression of GSTP1 as determined by Western blot analysis (data not shown). Cells were incubated for 24 hours before harvested in a mild NP-40 lysis buffer to maintain protein-protein interactions. GFP-tagged GSTP1 (2mg/ml) was immunoprecipitated from HCT116^{shGSTP1} lysates using GFP-Trap® -M beads (30µl). Proteins were resolved on a SDS-PAGE gel and analysed by Western blot analysis. Figure 4.22 demonstrates that transfection of GFP-tagged GSTP1 results in expression of a GSTP1 protein which corresponds to the molecular weight of a fusion between GSTP1 and GFP. This is evident from the lower migration of a GFP transfected plasmid compared to the higher migration of GFP-GSTP1 fused construct on the Western blot. The presence of GSTP1 protein in HCT116^{shGSTP1} cells expressed as a fusion between GSTP1 and GFP demonstrates that the silent mutations introduced into the open reading frame have successfully prevented silencing of the transfected construct, as no GSTP1 protein can be detected in GFP only transfected cells or in untreated HCT116^{shGSTP} cells. At resting levels, the expression of ETFα does not appear to differ after transfection of any GSTP mutational construct suggesting its expression is not transcriptionally regulated by GSTP1. Immunoprecipitation of GFP at resting levels results in a clean pull down of GSTP1 from HCT116^{shGSTP1} cells. Unfortunately no apparent ETFα could be co-immunoprecipitated with any polymorphic or catalytic variant of GSTP1, suggesting that ETFα is not post-translationally regulated by GSTP1. In order to determine whether a protein interaction could be detected after cellular stress, cells were treated with cisplatin (50µM) or etoposide (25µM)

for 6 hours in order to induce apoptosis in HCT116^{shGSTP1} cells. Treatment with either compound did not induce transcriptional changes in ETFα expression upon transfection with GFP-tagged constructs. ETFα expression could not be detected after co-immunoprecipitation of GFP after treatment with either apoptosis inducing agents, indicating that, in HCT116 cells, GSTP1 is unlikely to regulate ETFα.

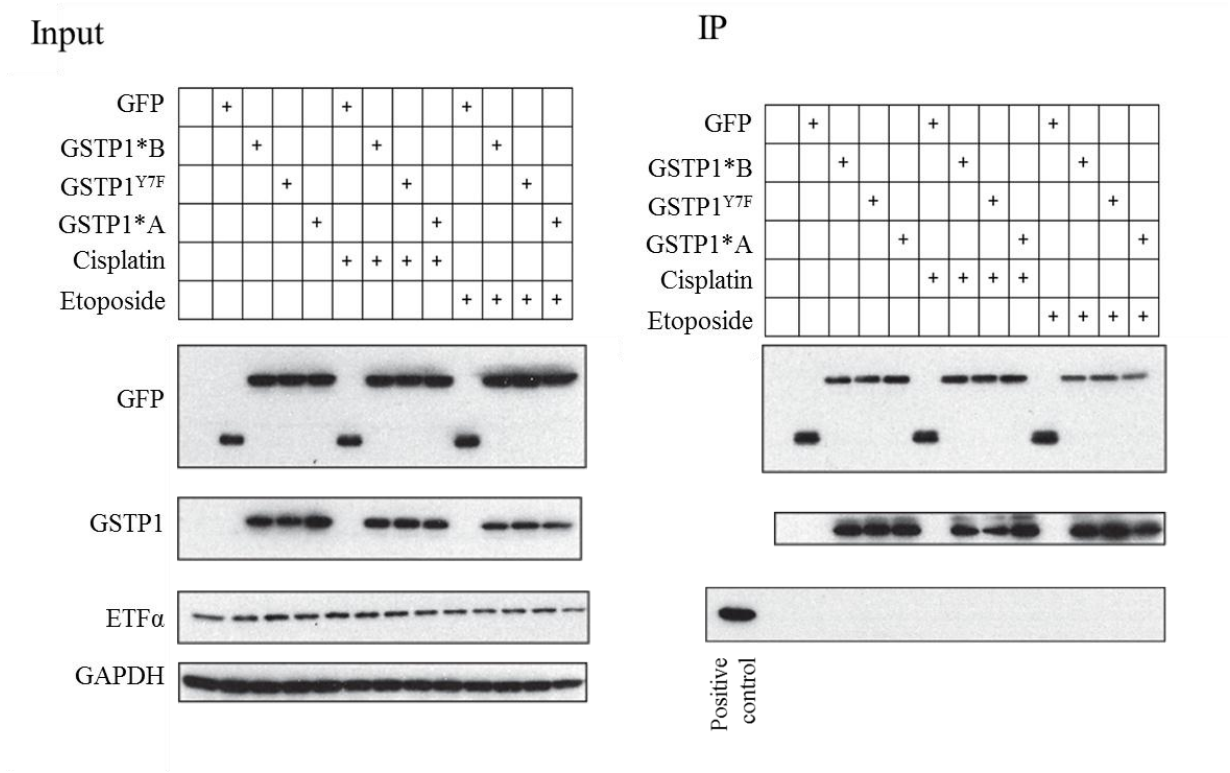


Figure 4.22. Immunoprecipitation of transfected GFP-tagged GSTP1 constructs from HCT116 cells.

HCT116^{shGSTP} cells were transfected GFP-tagged GSTP1 constructs harbouring *A or *B polymorphism or Y7F (non-catalytic GSTP1) mutation. Each construct contained silent mutations within the shRNA binding region to allow expression of GSTP1. After transfection (24 hours), cells were treated with cisplatin (50μM) or etoposide (25μM) for 6 hours. Cells were harvested and lysates (2mg/ml) were incubated with GFP-Trap® beads for 2 hours at 4°C to immunoprecipitate GFP-tagged constructs. Proteins isolated from beads were resolved on a SDS-PAGE gel and analysed by Western blotting. Blots are representative of 3 independent experiments.

In summary, GSTP1 does not appear to be intrinsic in catalysing the S-glutathionylation of proteins in HCT116 cells which is in contrast to previously published data (Townsend et al., 2008a). In fact, transient knockdown of GSTP1 in HCT116 cells induces the S-glutathionylation of a number of proteins; however, this phenotype was not apparent in cells stably silenced for GSTP1 and therefore may not be a universal function of GSTP1. Although the S-glutathionylation of a number of mitochondrial proteins was increased when GSTP1 was silenced transiently, no mitochondrial function could be attributed to GSTP1 in subsequent studies. Despite this, GSTP1 may have a role in mediating sulfhydryl homeostasis and redox control as transient or stable silencing of GSTP1 increases levels of glutathione disulphide in HCT116 cells. As previously demonstrated, GSTP1 is involved, although not necessary, in the proliferation of HCT116 cells (Dang et al., 2005); this phenotype is examined further in Chapter 5.

5. Chapter 5: Identification of GSTP1 mediated cytoprotection through chemical inhibition and microarray analysis

Introduction

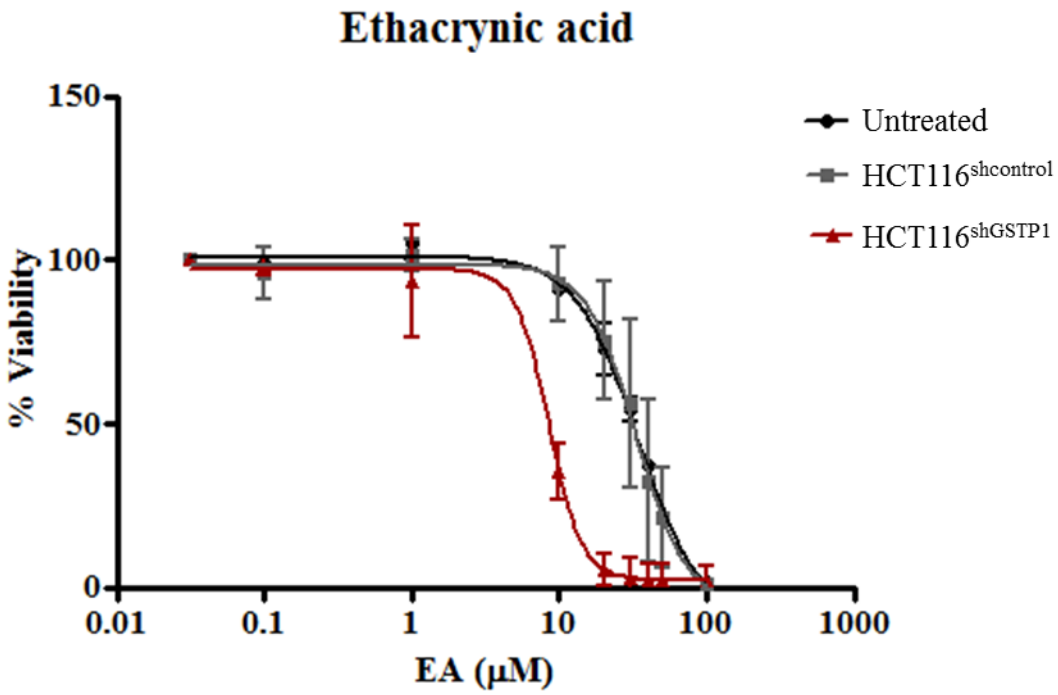
Chapter 4 examined the function of GSTP1 in relation to a specific post translational modification in the human colon carcinoma cell line, HCT116. Although GSTP1 did not appear to have a clear role in protein S-glutathionylation in HCT116 cells, it was evident that silencing GSTP1, transiently or stably, induced differences in phenotype particularly in relation to cell proliferation and oxidative stress. This chapter aims to assess the different pathways mediated by GSTP1 through the use of chemical inhibition and microarray analysis. The chapter will also examine the role of GSTP1 in determining cellular resistance to a range of anticancer compounds in order to identify potential pathways of GSTP1-mediated resistance.

Results

5.1 GSTP1 mediates resistance to ethacrynic acid but not to platinum compounds

Initial experiments were designed to validate the use of HCT116^{shGSTP1} cells as a viable tool in cytotoxic assays using a well-characterised GSTP1 substrate, ethacrynic acid. Ethacrynic acid (EA) binds to the Cys47 residue on GSTP1, thereby affecting its ability to bind to other substrates (Phillips and Mantle, 1993). Inhibiting GSTP1 with EA increases the sensitivity of tumour cells to cisplatin (Wang et al., 2007) and can induce apoptosis in human Jurkat T cells

(McCaughan et al., 1994). Although proven unsuccessful in clinical trials due to its diuretic effects, analogues of ethacrynic acid have been synthesised and show anti-proliferative effects in tumour cells associated with inhibition of GSTP1 (Yang et al., 2010). In this study, IC_{50} curves using EA were generated to determine the effect of GSTP1 knockdown in HCT116 cells and used as a positive control for other anticancer agents. The hypothesis assumes that GSTP1 knockdown cells would be more sensitive to EA treatment, owing to the specificity of EA for GSTP1. A number of $HCT116^{shcontrol}$ and $HCT116^{shGSTP1}$ clone cells, generated as described in Chapter 4, were incubated with EA over a 72 hour period. As predicted, treatment of HCT116 cells with EA induces cell death in a dose-dependent manner which is greatly enhanced in the absence of GSTP1, as evident from a left-hand shift in the IC_{50} curves in EA-treated $HCT116^{shGSTP1}$ cells (Figure 5.1). EA treatment was tested in multiple GSTP1 shRNA clones and therefore the effect appears to be indicative of the absence of GSTP1 and not as a result of cell transduction. The large difference in IC_{50} as a result of specifically targeting GSTP1 is a positive control which provides confidence in pursuing further cytotoxicity studies targeting specific pathways or regulatory processes dependent on GSTP1.



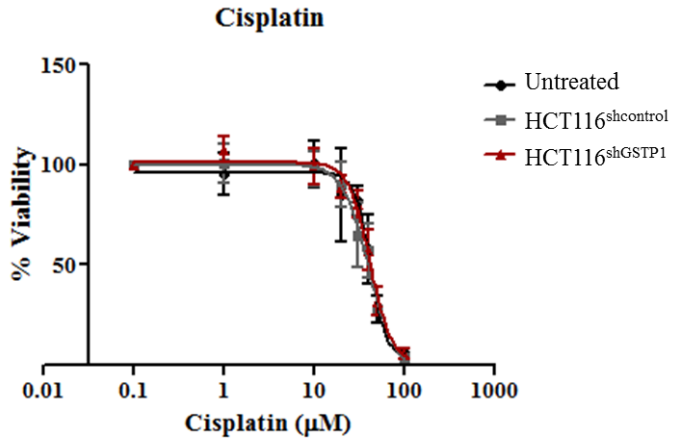
IC50 (95% confidence intervals)		
Untreated	HCT116^{shcontrol}	HCT116^{shGSTP1}
34.1 (29.6, 39.11)	33.1 (25.9, 42.2)	8.5 (7.3, 9.9)

Figure 5.1. IC_{50} curves of ethacrynic acid treated HCT116 cells stably silenced for GSTP1.

HCT116 (n=3), HCT116^{control} (n=3) and HCT116^{shGSTP1} (n=6) cells were seeded into a 96 well plate at 1500 cells/well and incubated for 24 hours. Cells were incubated with different concentrations of ethacrynic acid (EA). Cell viability was determined using the ATP assay 72 hours after EA treatment. The IC50 values are shown in the table above. Values are represented as mean ± standard deviation.

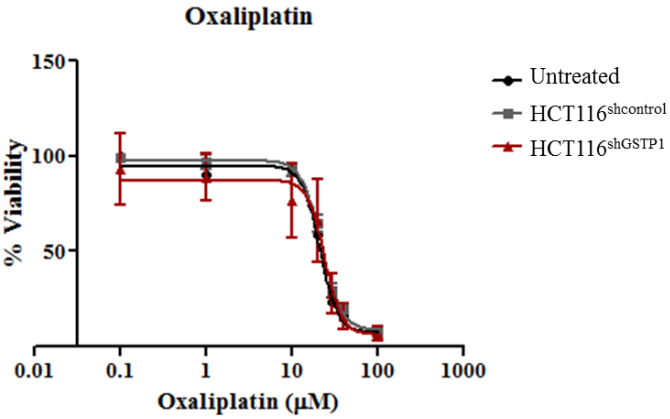
Initial studies involving GSTP1 mediated signalling and compound resistance in HCT116 cells examined the cytotoxicity of two well-characterised platinum compounds, cisplatin and oxaliplatin. GSTP1 can facilitate the GSH conjugation of platinum compounds and therefore overexpression of GSTP1 is thought to play a key role in platinum resistance in cancer chemotherapy (Goto et al., 1999). The hypothesis is that in HCT116 cells silenced for GSTP1, one would expect an increase in platinum sensitivity due to reduced conjugation with GSH. Contrary to this, cytotoxicity analysis of both platinum compounds in HCT116 cells did not yield any significant difference in IC_{50} curves when GSTP1 was silenced (Figure 5.2). Therefore in HCT116 cells, GSTP1 does not appear to play a role in mediating platinum resistance. The effect is probably not caused by a polymorphism of GSTP1 in HCT116 cells, as described in Chapter 4, as Peklak-Scott et al demonstrated that this polymorphism increased the conjugation of GSH to cisplatin 80-90% over its wild-type counterpart and that this increased rate of GSH conjugation to cisplatin did not increase the resistance of cells to cisplatin treatment, speculating that GSTP1 must protect cells from cisplatin by other means. (Peklak-Scott et al., 2008). The role of GSTP1 in mediating oxaliplatin resistance is somewhat contradictory. Despite a lack of correlation between GST activity and oxaliplatin treatment demonstrated by some groups (Arnould et al., 2003), others have suggested polymorphisms in GSTP1 (Ile105Val) may be of use in predicting survival of patients with advanced colorectal cancer whilst receiving 5-fluorouracil/oxaliplatin treatment (Stoehlmacher et al., 2002). If the latter was true in this study, $HCT116^{shGSTP1}$ cells should show a marked reduction in resistance to oxaliplatin, which is not the case.

A)



IC50 (95% confidence intervals)		
Untreated	HCT116^{shcontrol}	HCT116^{shGSTP1}
41.8 (36.0, 48.4)	40.4 (34.5, 47.4)	41.7 (38.8, 44.4)

B)



IC50 (95% confidence intervals)		
Untreated	HCT116^{shcontrol}	HCT116^{shGSTP1}
21.6 (20.1, 23.0)	22.7 (21.7, 23.7)	24.5 (21.0, 28.7)

Figure 5.2. IC_{50} curves of cisplatin and oxaliplatin treated HCT116 cells stably silenced for GSTP1.

HCT116 (n=3), HCT116^{control} (n=3) and HCT116^{shGSTP1} (n=6) cells were seeded into a 96 well plate at 1500 cells/well and incubated for 24 hours. Cells were incubated with different concentrations of cisplatin **(A)** or oxaliplatin **(B)**. Cell viability was determined using the ATP assay 72 hours after platinum treatment. The IC50 values are shown in the tables above. Values are represented as mean ± standard deviation.

5.2 Elucidating GSTP1 mediated cytoprotection through a multi-compound cytotoxicity screen

GSTP1 is often found to be overexpressed in cancer cells and is associated with resistance to a wide range of anticancer and chemical treatments. It is unknown what the role of GSTP1 is in mediating resistance against such compounds, as many are not known substrates for GSTP1. The effect of GSTP1 on the cytotoxicity of EA is clearly evident in HCT116 cells but is less prominent with platinum compounds. As established in Chapter 4, the mechanism of GSTP1-mediated cytoprotection appears independent of JNK activity and therefore new insights are needed to determine the molecular pathways mediated by GSTP1 in HCT116 cells. In order to assess this, a cytotoxicity screen was performed using 31 compounds targeted against different molecular pathways and cellular processes. A full list of compounds and their modes of action/target are shown in Table 11. Differences in cytotoxicity between HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells may indicate as to the molecular pathways mediated by GSTP and provide novel insight into the metabolism of these compounds, highlighting a potential use for improved or targeted drug dosing depending on the GSTP1 expression of a tumour.

Compound	Mode of action/target
Carmustine	Alkylating agent – forms DNA crosslinks
Cyclophosphamide	Alkylating agent – forms DNA crosslinks
Lomustine	Alkylating agent – inhibits DNA synthesis
Cisplatin	Causes crosslinking of DNA
Cladribine	Adenosine deaminase
Tosedostat	Aminopepdidase
Anastrazole	Aromatase
Azacitidine	DNA methyltransferase
Gemcitabine	Ribonucleotide reductase
Doxorubicin	DNA topoisomerase II
XAV939	Tankyrase 1 and 2
Cyclopamine	Smoothened
GDC-0449	Smoothened
CI-994	Histone deacetylase 1 and 3
Vorinostat	Histone deacetylase 1 and 3
NVP-AUY922	Heat shock protein 90
17AAG	Heat shock protein 90
Roscovitine	Cyclin-dependent kinase
Everolimus	Mammalian target of rapamycin (mTOR) complex 1
LY294002	Phosphoinositide 3-kinases
Pim1 inhib2	Pim1
PLX4032	BRAF
Erlotinib	Epidermal growth factor receptor

NVP-AEW541	Insulin-like growth factor receptor 1
Vatalanib	Vascular endothelial growth factor receptor 1 Platelet-derived growth factor receptor
Docetaxel	Microtubule
Navitoclax	Bcl2/Bcl-xL
ABT888	Poly (ADP-ribose) polymerase
Thiotepa	DNA
Pazopanib	Vascular endothelial growth factor receptor 1,2 and 3 Platelet-derived growth factor receptor c-Kit

Table 11. Compounds targeting a number of cellular pathways and targets were used in a cytotoxicity screen against HCT116 cells silenced for GSTP1.

The table highlights each compound used in a cytotoxicity screen against $HCT116^{shGSTP1}$ cells and its mode of action. Where possible, more than one compound was designated to a particular molecular pathway/target.

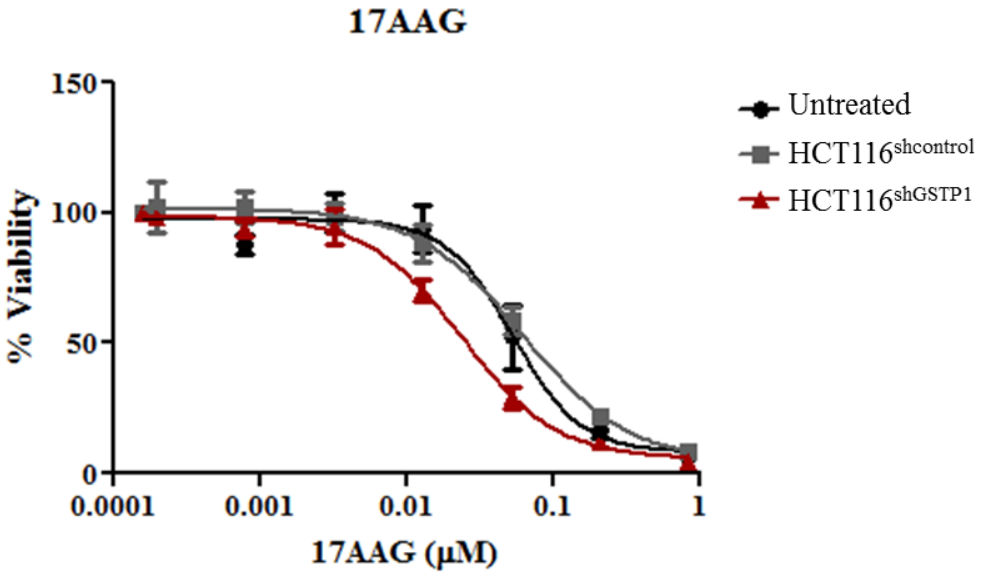
HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells were seeded at 1500 cells/well onto a 96 well plate and incubated with the compounds listed in Table 11 for 72 hours followed by a cytotoxicity assessment using the ATP assay. Any edge effect of the 96 well plates on cell viability was taken into account by incubating the same concentration of drug onto different areas of multiple 96 well plates. The range of drug concentration used was optimised for each compound in untreated HCT116 cells as a preliminary screen to ensure accurate readings of IC_{50} values. IC_{50} values were then generated for each compound in HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells and are highlighted in Table 12. Some compounds did not induce toxicity in HCT116 cells at the concentrations tested and so were omitted from the final analysis. These mostly included drugs such as anastrazole and XAV939, where the parent compound needs to be metabolised into its active component. As HCT116 cells do not contain a functioning cytochrome P450 system (Nakumura et al., 2003), it is understandable that these drugs did not produce a cytotoxic effect. Other compounds saw pronounced differences between untreated HCT116 cells and HCT116^{shcontrol} cells and therefore were also excluded. From the compounds tested, very few appeared to show any difference in sensitivity in HCT116^{shGSTP1} cells and of the drugs which did show differences in drug sensitivity, there are very few similarities in their mode of action. Doxorubicin, 17-AAG and GDC-0449 were taken for a second set of screening over an improved dose range.

	IC50 values (µM, plus 95% confidence intervals)		
Compound	Untreated	shRNA Control	shRNA GSTP1
GDC-0449	14.3 (11.2, 18.2)	17.2 (13.5, 22.0)	10.9 (8.5, 14.0)
Carmustine	46.4 (35.2, 61.0)	29.8 (22.1, 40.1)	28.8 (24.5, 34.0)
Lomustine	21.6 (18.0, 25.9)	17.6 (13.7, 22.7)	15.0 (11.8, 18.9)
Navataclax	6.4 (5.7, 7.2)	3.5 (2.9, 4.1)	3.7 (3.1, 4.4)
Gemcitibine	0.015 (0.014, 0.016)	0.014 (0.012, 0.016)	0.014 (0.011, 0.018)
Cyclopamine	7.3 (6.6, 8.0)	7.9 (6.7, 9.2)	6.5 (4.8, 8.8)
PLX0432	12.43 (9.4, 16.5)	17.0 (11.8, 24.4)	12.32 (7.6, 19.9)
Erlotinib	1.6 (1.3, 1.9)	1.6 (1.3, 1.9)	1.4 (1.2, 1,7)
17-AAG	0.092 (0.067, 0.013)	0.17 (0.11, 0.25)	0.043 (0.032, 0.058)
Azacitidine	13.7 (9.4, 19.9)	11.7 (5.9, 23)	8.1 (5.9, 11.2)
Doxorubicin	0.066 (0.05, 0.09)	0.11 (0.06, 0.23)	0.04 (0.03, 0.05)
CI-994	4.6 (3.8, 5.6)	4.6 (3.9, 5.5)	3.8 (3.1, 4.7)
LY294002	13.0 (9.6, 17.6)	10.4 (7.0, 15.4)	9.7 (7.2, 13.0)
NVP-AEW5411	0.35 (0.31, 0.39)	0.31 (0.22, 0.43)	0.26 (0.22, 0.3)

Table 12. IC_{50} values from a panel of anticancer compounds against HCT116 cells silenced for GSTP1.

HCT116, $HCT116^{control}$ and $HCT116^{shGSTP1}$ (n=4) cells were seeded into a 96 well plate at 1500 cells/well and incubated for 24 hours. Cells were incubated with a range of anticancer compounds for 72 hours before cell viability was determined using the ATP assay. Values are represented as mean of IC_{50} ± 95% confidence intervals.

In this second screen, only the Hsp90 inhibitor, 17-AAG cytotoxicity retained a significant increase in sensitivity, demonstrating a 2.25-2.6 fold increase in cytotoxicity sensitivity in HCT116^{shGSTP1} cells (Figure 5.3). Hsp90 chaperones a large of number of proteins involved in protein kinase signalling and transcription factors. 17-AAG is a geldanamycin analogue which binds to a conserved ATP site in the N-terminal domain of Hsp90, disrupting its chaperone complex (Whitesell and Lindquist, 2005, Whitesell et al., 1994). The extent to which GSTP1 may facilitate this is unclear as another Hsp90 inhibitor, the resorcinylic isoxazole amide NVP-AUY922, did not show any significant difference in cytotoxicity between HCT116^{shGSTP1} and control cells (Table 12) indicating that the effect of GSTP1 in mediating 17-AAG resistance may be drug specific. 17-AAG is metabolised primarily via the cytochrome P450 system (Egorin et al., 1998, Banerji et al., 2005) but can also be metabolised by NQO1 into its hydroquinone derivative (Guo et al., 2005). Whether changes in NQO1 activity as a result of GSTP1 silencing may account for differences in metabolism of 17-AAG has yet to be determined as GSTP1 has not been shown to aid directly in the metabolism of 17-AAG.



IC50 (95% confidence intervals)		
Untreated	HCT116^{shcontrol}	HCT116^{shGSTP1}
0.054 (0.042, 0.068)	0.063 (0.048, 0.083)	0.024 (0.021, 0.029)

Figure 5.3. IC_{50} curve of 17-AAG treatment in HCT116 cells silenced for GSTP1.

HCT116 HCT116^{control} and HCT116^{shGSTP1} (n=4) cells were seeded into a 96 well plate at 1500 cells/well and incubated for 24 hours. Cells were incubated with different concentrations of 17-AAG and cell viability was determined using the ATP assay 72 hours later. The IC50 values are shown in the table above in µM. Values are represented as mean ± standard deviation.

5.3 Gene expression analysis of GSTP1 silencing in HCT116 cells

The cytotoxicity screen produced few compounds which showed differential toxicity when GSTP1 is silenced, proving difficult to identify novel cellular pathways associated with GSTP1 mediated cytoprotection. In addition to the cytotoxicity screen, mRNA profiles were determined from HCT116, HCT116^{shcontrol} and HCT116^{shGSTP1} cells using the Illumina® HumanHT-12 v4 Expression BeadChip array. Microarray profiling of HCT116^{shGSTP1} cells may shed light on novel pathways associated with GSTP mediated cytoprotection and aid in determining a cellular mechanism behind the observations made in Chapter 4 related to cell proliferation and oxidative stress. The microarray profiling demonstrated good clustering of biological samples within each genotype (Figure 5.4A), whilst showing little variation in signal (Figure 5.4B). As a positive control, GSTP1 was found to be the most down-regulated gene in HCT116^{shGSTP1} cells. Unfortunately, we observe that the control plasmid has had an effect on gene expression when compared to untreated HCT116 cells as evident from the clustering analysis (Figure 5.4A). To account for differences in gene expression due to off target effects mediated by the control plasmid, gene expression profiles of 2 separate HCT116^{shGSTP1} clones were compared against both untreated HCT116 and HCT116^{shcontrol} mRNA profiles to generate 4 sets of gene expression profiles. Gene sets were then compared against each other to look for similarly regulated gene profiles. In practice, this is achieved through a four-way comparison; untreated vs HCT116^{shGSTP1} clone 1, untreated vs HCT116^{shGSTP1} clone 2, HCT116^{shcontrol} vs HCT116^{shGSTP1} clone 1 and HCT116^{shcontrol} vs HCT116^{shGSTP1} clone 2 (Figure 5.5). By comparing the data as such, the level of false positives due to off-target effects by both control shRNA and individual GSTP1 shRNA plasmids are minimised. The gene expression profiles of untreated HCT116 cells against HCT116^{shcontrol} cells were also compared to the extent of any off target effects (Table 15).

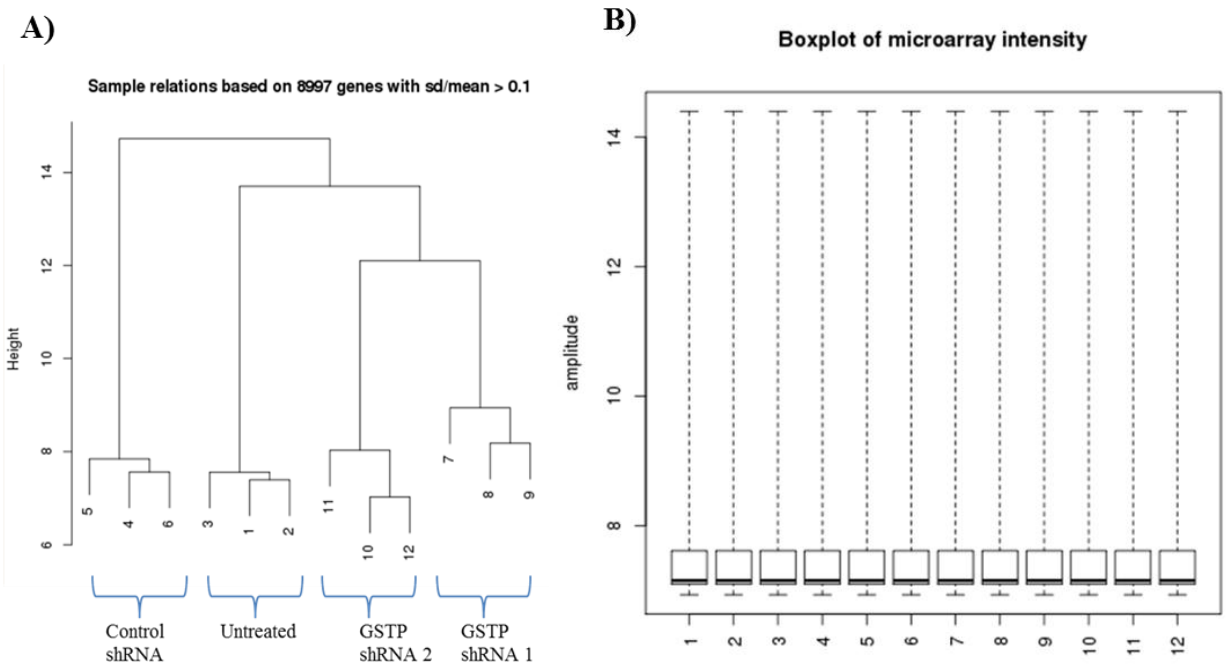


Figure 5.4. Analysis of microarray profiling of HCT116 cells silenced for GSTP1.

mRNA from HCT116, HCT116^{shcontrol} and two individual HCT116^{shGSTP1} clones were profiled by microarray analysis. Biological mRNA triplicates were taken from each genotype and amplified to aRNA before analysed using the Illumina® HumanHT-12 v4 Expression BeadChip array. **A)** Cluster analysis of samples shows accurate grouping of biological samples for each genotype. **B)** Boxplot intensity profiles demonstrate little variation in signal between samples tested.

Typically, the cut-off value for biologically significant changes in gene expression is greater than a two-fold difference. However, in order to examine statistically significant changes in gene expression across the number of comparisons made, the cut-off for a change in gene expression was decreased to 1.2 fold. This takes into account statistically significant changes in gene expression and not just biological changes. Initial analysis of the raw microarray data as shown in Table 13 and Table 14 shows a number of genes that differ in mRNA expression in HCT116^{shGSTP1} cells from HCT116 untreated or HCT116^{shcontrol} cells, although very few

genes show a large difference in fold expression from control cells. It must be acknowledged that some differentially expressed genes in HCT116^{shGSTP1} cells in comparison to HCT116^{shcontrol} mRNA may be as a result of off-target effects from the control plasmid. This is evident from examining gene expression profiles from HCT116^{shcontrol} and HCT116 untreated mRNA profiles (Table 15). For example, *CAV1* mRNA is decreased 3- to 4-fold in HCT116^{shGSTP1} cells when compared to HCT116^{shcontrol} cells but is upregulated 2.4-fold in HCT116^{shcontrol} cells compared to untreated HCT116 cells and therefore the actual difference in gene expression may not be physiologically significant. In order to circumvent this potential problem, gene expression profiles were compared against each other and similarly expressed (i.e. upregulated or downregulated) genes across each four-way comparison were identified (Table 16). For example, *GSTP1* is downregulated in each of the four-way comparisons described in Table 16 and is therefore regarded as a significant result, acting as a positive control in this regard. Understandably, this protocol may lead to the loss of gene targets which may have a true effect but as a result in gene expression differences between control cells, have been omitted. For example, in HCT116^{shGSTP1} clone 1 cells, *VGF* is upregulated 2.6 fold in comparison to HCT116^{shcontrol} cells, 1.6 fold in comparison to untreated cells and is upregulated 1.5 fold in HCT116^{shGSTP1} clone 2 cells compared to HCT116^{shcontrol} cells. However, as it is not differentially expressed between HCT116^{shGSTP1} clone 2 cells and untreated HCT116 cells, it was omitted from the final analysis.

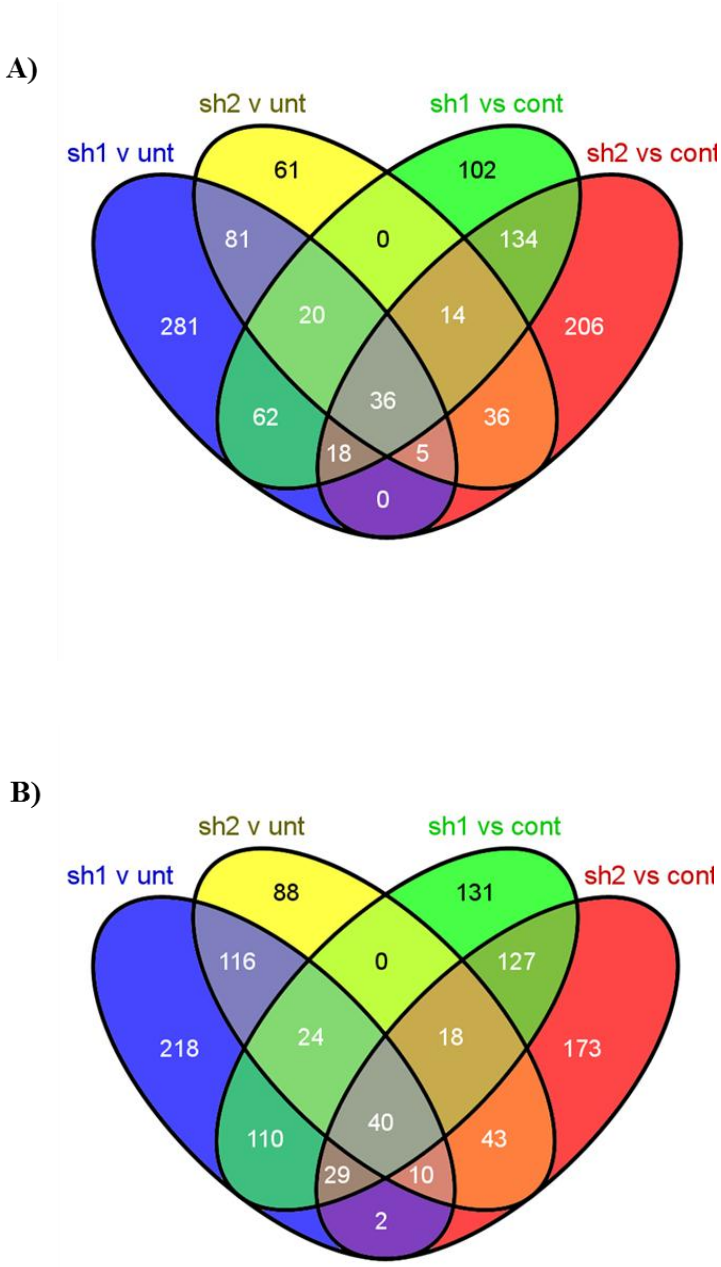


Figure 5.5. Venn diagrams showing comparative gene expression analysis of GSTP1 silencing in HCT116 cells.

mRNA expression profiles from HCT116, HCT116shcontrol and 2 individual HCT116shGSTP1 clones were compared against each other in a four-way Venn diagram. The numbers in each segment depict the number of simultaneously upregulated genes **(A)** and downregulated genes **(B)** between each comparison. Unt, untreated HCT116 cells; cont, $HCT116^{shcontrol}$; sh1, $HCT116^{shGSTP1}$ clone 1; sh2, $HCT116^{shGSTP1}$ clone 2.

In total, 36 genes were found to be similarly upregulated and 40 genes downregulated in HCT116^{shGSTP1} cells (Figure 5.5). The top 10 genes differentially expressed in HCT116^{shGSTP1} cells are shown in Table 16, with their range in fold differences across each comparison shown. Very few genes had any significant fold change in mRNA expression across each comparison, with the exception of *GSTP1*, which serves as a positive control. From the data generated, it was difficult to assess the biological response to GSTP1 silencing in HCT116 cells due to a lack of uniform gene sets conferring a similar biological response. Whilst silencing of GSTP1 appears to induce the upregulation of sulfotransferases and NQO1 mRNA expression, the fold difference in mRNA expression may not be biologically significant. What may be important is the upregulation of these genes in relation to a general cellular pathway or process which is discussed later in the chapter.

Top 20 downregulated genes							
shGSTP1 clone 1 vs Untreated		**shGSTP1 clone 2 vs Untreated**		**shGSTP1 clone 1 vs shcontrol**		**shGSTP1 clone 2 vs shcontrol**	
Gene	Fold difference	Gene	Fold difference	Gene	Fold difference	Gene	Fold difference
GSTP1	-13.7016	GSTP1	-10.1243	GSTP1	-12.5088	GSTP1	-9.24294
TRIB3	-3.06358	IFITM1	-2.42911	CYP24A1	-4.68101	CAV1	-4.0235
PCK2	-2.91263	SQLE	-2.02962	S100A10	-3.35066	CYP24A1	-3.40112
DDIT3	-2.86761	TSC22D3	-2.00164	CAV1	-3.31936	CAV2	-3.11606
TNFRSF6B	-2.66571	TM4SF18	-1.94673	CPA4	-3.30832	CAV1	-3.09288
ASNS	-2.56581	MKX	-1.84514	S100A10	-2.74879	CPA4	-3.02439
MKNK2	-2.51069	UPP1	-1.83989	CAV2	-2.51733	CAV2	-2.77899
DDIT4	-2.40636	TSC22D3	-1.79255	CAV1	-2.486	GPR110	-2.40488
LOC652097	-2.29801	CNTNAP2	-1.72482	F3	-2.42727	F3	-2.26335
TNFRSF6B	-2.18582	RBCK1	-1.70943	KRT81	-2.38986	KRT81	-2.06352
ASNS	-2.17479	MKNK2	-1.70175	ATG12	-2.36186	F3	-2.05669
ATG12	-2.17424	TM4SF18	-1.68122	LOC652097	-2.279	SQLE	-2.028
SLC2A3	-2.1417	ARHGEF2	-1.65596	LOC647349	-2.2714	PTPN1	-1.97687
MKX	-2.1393	LOC730167	-1.65155	NP	-2.23517	GPR110	-1.97143
IFITM1	-2.12686	TRIB3	-1.64458	BCAR3	-2.17099	GPR110	-1.95635
LOC730167	-2.12377	PCK2	-1.62361	CAV2	-2.13388	BCAR3	-1.91522
ARHGEF2	-2.1129	ANXA3	-1.61315	MKNK2	-2.13014	LOC728285	-1.88141
PTK2	-2.09527	ADAM19	-1.60416	AP3S1	-2.09271	SLC1A3	-1.86505
ID1	-2.09469	HSPH1	-1.58939	PTPN1	-2.06786	LOC647349	-1.73658
S100A10	-2.09187	KLF2	-1.57796	F3	-2.01684	NP	-1.73423

Table 13. Top 20 downregulated genes in GSTP1 silenced HCT116 cells when compared to both untreated and control shRNA treated HCT116 cells.

mRNA expression profiles from 2 individual HCT116^{shGSTP1} clones were compared against mRNA expression profiles from HCT116 (untreated) and HCT116^{shcontrol} cells (n=3). Probes that exhibited an adjusted *P* value of <0.05 are expressed.

Top 20 upregulated genes							
shGSTP1 clone 1 vs Untreated		**shGSTP1 clone 2 vs Untreated**		**shGSTP1 clone 1 vs shcontrol**		**shGSTP1 clone 2 vs shcontrol**	
Gene	Fold difference	Gene	Fold difference	Gene	Fold difference	Gene	Fold difference
SCG2	2.863652	RGS2	2.785131	FXR1	5.000266	ANXA10	5.371668
HSPA1B	2.765232	0	2.031254	SCG2	4.300566	FXR1	4.795362
SLC4A7	2.533093	HSPA1B	1.853671	FXR1	3.909493	RGS2	4.412696
SYTL2	1.928746	SYTL2	1.686146	LOC100132797	3.466396	FXR1	3.751463
DNAJB1	1.895632	F2RL1	1.616699	ACSL5	2.829557	LOC100132797	3.253591
FAM122B	1.856638	SLC4A7	1.579626	S100A16	2.61553	ACSL5	2.703254
DHRS2	1.851713	SNORA12	1.579433	VGF	2.609363	DDIT3	2.474316
DNAJA1	1.827864	AKAP12	1.561662	RGS2	2.370608	0	2.314096
F2RL1	1.818131	DNAJB1	1.533974	DHRS2	2.355288	TRIB3	2.285847
SLC4A7	1.800397	ANKRD37	1.527654	HSPH1	2.095961	S100A16	2.26521
DNCL1	1.779618	MIR1978	1.526641	ANXA10	2.095487	ACSL5	2.210474
CASP4	1.76604	DYNLL1	1.521144	CASP4	2.056673	DDIT4	2.17986
F2RL1	1.760198	GPR177	1.51515	PDE4B	1.971111	HAS3	2.158181
DYNLL1	1.726351	HMGCS1	1.509488	ACSL5	1.95556	PCK2	2.009696
GCLM	1.689124	LOC392437	1.498877	STXBP6	1.922043	ASNS	1.998808
FABP6	1.670221	RDH10	1.490909	ALDH1L1	1.90011	SLC2A3	1.987838
MRPL33	1.650463	DHRS3	1.484901	MCM5	1.890673	ALDH1L1	1.930505
SEPX1	1.626125	TDP1	1.473749	SULT1A1	1.8503	LOC100130516	1.841752
LOC100129673	1.617534	RNY1	1.44571	SULT1A4	1.831269	MCM5	1.838512
EPDR1	1.606632	PCBD1	1.445189	0	1.797543	SLC20A1	1.834155

Table 14. Top 20 upregulated genes in GSTP1 silenced HCT116 cells when compared to both untreated and control shRNA treated HCT116 cells.

mRNA expression profiles from 2 individual HCT116^{shGSTP1} clones were compared against mRNA expression profiles from HCT116 (untreated) and HCT116^{shcontrol} cells (n=3). Probes that exhibited an adjusted *P* value of <0.05 are expressed.

Genes upregulated in control shRNA vs untreated HCT116 cells		**Genes downregulated in control shRNA vs untreated HCT116 cells**	
Gene	Fold difference	Gene	Fold difference
CPA4	3.724755	ANXA10	-2.09058
HSPA1B	2.886556	TRIB3	-1.91045
CAV1	2.840511	FXR1	-1.8274
CYP24A1	2.692376	IFITM1	-1.74687
CAV1	2.361086	PCK2	-1.70618
CAV2	2.325084	DDIT3	-1.66086
KRT81	2.277799	LOC100132797	-1.63645
CAV2	2.233047	SLC2A3	-1.60846
DNAJB1	2.04252	FXR1	-1.59934
F3	1.986304	ASNS	-1.56559
DNAJA1	1.836899	ACSL5	-1.46811
LOC728285	1.831077	MKX	-1.37163
F3	1.807781	HKDC1	-1.34448
GPR110	1.750518	TSC22D3	-1.2278
BCAR3	1.712693	PSAT1	-1.2203

Table 15. mRNA expression profile comparison between control shRNA treated HCT116 cells and untreated HCT116 cells.

Of the genes identified, MAP kinase interacting serine/threonine kinase 2 (*MKNK2 or MNK2*) expression was validated in a rtPCR reaction as it appeared to be one of the more downregulated genes identified from the comparison analysis (Table 16). MKNK2 is a member of the calcium/calmodulin-dependent protein kinases (CAMK) serine/threonine protein kinase family and is activated primarily through the ERK Kinase signalling pathways (Fukunaga and Hunter, 1997, Waskiewicz et al., 1997). MKNK2 is involved in the phosphorylation of the eukaryotic initiation factor 4E (eIF4E) and therefore plays an important role in translation and proliferation (Waskiewicz et al., 1997, Joshi et al., 2009). Activation of eIF4E by MKNK2 results in enhanced tumour proliferation in human glioma cells, which is attenuated when MKNK2 is silenced (Ueda et al., 2010). Silencing of GSTP1 significantly reduces the level of MKNK2 expression in HCT116 cells in two HCT116^{shGSTP1} clones as determined by rtPCR (Figure 5.6). Although there is a statistically significant difference between untreated HCT116 and HCT116^{shcontrol} cells, indicative of an effect mediated by the negative control plasmid, the change in expression is not great, with MKNK2 expression 87% to that of untreated HCT116 cells and therefore may have little biological significance. These data supports the means by which the microarray was examined and leads to an interesting hypothesis in that a reduction in MKNK2 expression as a result of GSTP1 silencing could potentially lead to a reduction in eIF4E phosphorylation resulting in reduced cell growth and proliferation, which may explain the observed decrease in proliferation in HCT116^{shGSTP1} cells.

Genes upregulated in GSTP1 shRNA HCT116 cells			**Genes downregulated in GSTP1 shRNA HCT116 cells**		
Gene	Name	Fold difference	Gene	Name	Fold difference
TSC22D1	TSC22 domain family	1.2 to 1.6	GSTP1	Glutathione S-transferase Pi 1	-9.2 to -13.7
MIR1978	microRNA 1978	1.4 to 1.5	MKNK2	MAP kinase interacting serine/threonine kinase 2	-1.4 to -2.5
RGS2	Regulator of G-protein Signalling 2	1.5 to 4.4	PTK2	Protein tyrosine kinase 2	-1.3 to -2.1
SULT1A1	Sulfotransferase 1A1	1.4 to 1.9	KLF2	Kruppel-like factor 2	-1.3 to -2.1
SULT1A4	Sulfotransferase 1A4	1.3 to 1.8	NP	Nucleoside phosphorylase	-1.5 to -2.2
S100A16	S100 calcium binding protein A16	1.3 to 2.6	ATP6V1B2	ATPase, H+ transporting, lysosomal V1 subunit B2	-1.4 to -1.9
TDP1	Tyrosyl-DNA phosphodiesterase 1	1.4 to 1.7	CYP24A1	Cytochrome P450 24A1	-1.3 to -4.7
FXR1	Fragile X Mental Retardation	1.4 to 5.0	TM4SF18	Transmembrane 4 L six family member 18	-1.2 to -1.9
ZDHHC6	Zinc finger, DHHC-type containing 6	1.3 to 1.5	ACAT1	Acetyl-Coenzyme A acetyltransferase 1	-1.4 to -1.7
NQO1	NAD(P)H dehydrogenase, quinone 1	1.2 to 1.8	AP3S1	Adaptor-related protein complex 3, sigma 1	-1.3 to -2.1

Table 16. Gene expression data of mRNA profiles from GSTP1 silenced HCT116 cells

mRNA expression profiles from 2 individual HCT116^{shGSTP1} clones were compared against mRNA expression profiles from HCT116 (untreated) and HCT116^{shcontrol} cells (n=3) in a four-way comparison as described in Figure 5.5. The profiles were collaborated and the table shows genes which were either upregulated or downregulated similarly across each set of comparisons. The values show the range in fold difference of gene expression across each comparison. Probes that exhibited an adjusted *P* value of <0.05 are expressed.

5.4 Enrichment analysis of microarray studies

In addition to examining individual changes in mRNA expression, data generated from the microarray was enriched to determine common molecular pathways or processes as a result of GSTP1 silencing in HCT116 cells. Gene enrichment of the data is separated into two analysis - cellular processes and cellular pathways - with two HCT116^{shGSTP1} clones compared against HCT116^{shcontrol} and untreated HCT116 mRNA profiles. HCT116^{shcontrol} ontology pathways were also compared against untreated HCT116 pathways to assess any off-target effects. A full list of statistically significant pathways and processes are provided in the Appendices (II, III, IV, V). Pathways and processes which did not prove statistically significant but occur across multiple comparisons (as described in Figure 5.5) are also included in the analysis.

Although many cellular processes are altered in individual HCT116^{shGSTP1} clone comparisons, very few processes are consistently exhibited across all possible comparisons, i.e., show the same pathway and process changes in each HCT116^{shGSTP1} clone compared to both HCT116^{shcontrol} and untreated HCT116 cells. For example, silencing of GSTP1 in HCT116 cells induces the upregulation of a number of cellular processes related to hypoxia and oxidative stress, with the induction of GSTK1, MGST2 and peroxiredoxin gene expression common across most comparisons. However, this process is not upregulated in one particular comparison, HCT116^{shGSTP1} clone 2 vs HCT116^{shcontrol} cells and therefore it is uncertain if this pathway is of biological significance. However, changes in oxidative response pathways may contribute to explaining why transient or stably silencing GSTP1 in HCT116 cells induces levels of glutathione disulphide, as no significant changes in the mRNA of any GCL subunits or GS could be detected from the microarray.

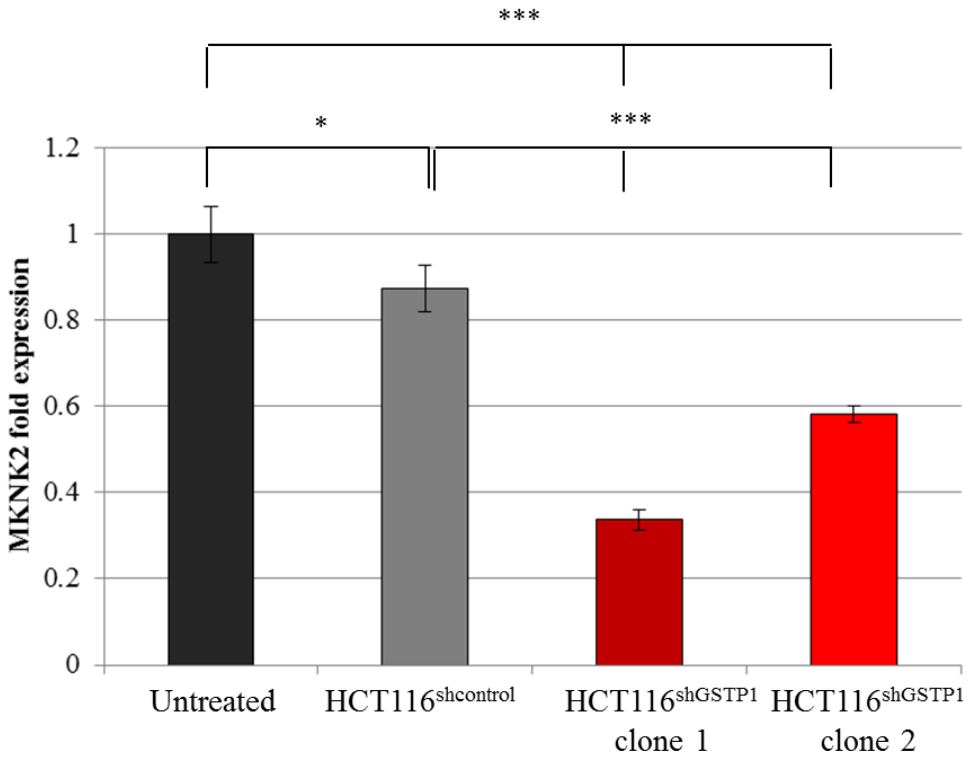


Figure 5.6. MKNK2 mRNA expression in GSTP1 silenced HCT116 cells.

HCT116 HCT116^{control} and HCT116^{shGSTP1} cells (n=3) were analysed for MKNK2 expression using rtPCR (Taqman). RNA (1µg) was synthesised to cDNA, diluted 1:80 and used in a duplex Taqman reaction using probes targeting MKNK2 and 18S as an endogenous loading control. ΔΔCt values were determined from the analysis and are displayed as percentage of expression in comparison to untreated HCT116 cells. Values are represented as mean ± standard deviation, where ***P <0.001 and *P <0.05.

Silencing of GSTP1 does lead to the induction of a number of genes related to protein folding and is common across all comparisons. This is evident from the upregulation of a number of Hsp genes such as Hsp90 and Hsp70 and, interestingly, the upregulation of glutaredoxin. However, protein folding can be categorised to a large number of compartments, such as the endoplasmic reticulum and cytoplasm and so it is difficult to assess whether this is a general event or specific to certain subcellular fractions. There is also some upregulation of these pathways in $HCT116^{shcontrol}$ cells when compared against untreated HCT116 cells and therefore the effect may be less significant. Another interesting find from examination of the pathway analysis is the upregulation of a number of pathways relating to fatty acid and catecholamine metabolism. This is largely due to the upregulation of a number of sulfotransferases in $HCT116^{shGSTP1}$ cells as described previously and in Table 16. Although these pathways are upregulated at varying degrees across all comparisons, they may suggest a common mechanism by which GSTP1 may mediate cell regulation.

As described in Chapter 4, transient or stable silencing of GSTP1 results in reduced proliferation of HCT116 cells. Examination of the enrichment data demonstrates a reduction in a large number of pathways related to cytoskeleton development and cell adhesion in $HCT116^{shGSTP1}$ cells. A list of the cellular processes related to cytoskeleton modelling is highlighted in Table 17. Although a reduction in cell adhesion pathways can be detected in $HCT116^{shcontrol}$ cells in comparison to untreated HCT116 cells, there is a large coverage of cellular processes related to cytoskeletal modification in $HCT116^{shGSTP1}$ cells that clearly demonstrate a reduction in processes associated with cell mobility. This is indicative of a mechanism which fits well with our observed data from Chapter 4 and with data previously published demonstrating the role of GSTP1 in cellular proliferation (Dang et al., 2005).

Down regulation of cellular processes in HCT116^{shGSTP1} cells							
shGSTP1 clone 1 vs Untreated		**shGSTP1 clone 2 vs Untreated**		**shGSTP1 clone 1 vs shcontrol**		**shGSTP1 clone 2 vs shcontrol**	
Process	*P* value	Process	*P* value	Process	*P* value	Process	*P* value
Cytoskeleton - Spindle microtubules	5.06E-03	Cytoskeleton ,Regulation of cytoskeleton rearrangement	2.44E-06	Cytoskeleton - Actin filaments	8.54E-06	Cytoskeleton - Spindle microtubules	1.03E-05
		Cell adhesion - Integrin-mediated cell-matrix adhesion	3.61E-05	Cell adhesion - Integrin-mediated cell-matrix adhesion	1.68E-05	Cytoskeleton - Intermediate filaments	5.95E-05
		Cytoskeleton - Intermediate filaments	2.17E-03	Cell adhesion - Cadherins	9.31E-05	Cytoskeleton - Cytoplasmic microtubules	7.85E-05
		Cytoskeleton - Actin filaments	3.36E-03	Cytoskeleton - Regulation of cytoskeleton rearrangement	1.23E-04	Cytoskeleton - Regulation of cytoskeleton rearrangement	1.79E-04
		Cytoskeleton - Cytoplasmic microtubules	1.08E-02	Cell adhesion - Cell junctions	3.71E-04	Cell adhesion - Integrin-mediated cell-matrix adhesion	2.68E-04
		Cell adhesion - Cell junctions	1.25E-02			Cytoskeleton - Actin filaments	7.28E-04
						Cell adhesion - Cell junctions	4.16E-03

Table 17. Enrichment analysis of downregulated processes related to cytoskeleton remodelling in GSTP1 silenced HCT116 cells.

Cellular processes related to mRNA profiles from HCT116, HCT116^{shcontrol} and 2 HCT116^{shGSTP1} clones were examined using Metacore software (Genego) and examined for similar ontology pathways. The table demonstrates downregulation of cellular processes related to cytoskeleton remodelling in HCT116^{shGSTP1} cells.

In summary, chemical inhibition and targeting of molecular pathways leads to the inclination that GSTP1 may mediate the effects of a Hsp90 inhibitor, 17-AAG. Despite a lack of uniformity with other Hsp inhibitors, the role of GSTP1 in relation to the efficacy of 17-AAG may be an important determinant in assessing resistance in tumours. Microarray analysis of cells silenced for GSTP1 showed a promising correlation between their reduced proliferation and a number of pathways relating to changes in cellular cytoskeletal processes which supports our observations made from previous chapters. Furthermore, reduced MKNK2 activity in HCT116^{shGSTP1} cells may prove one mechanism by which cellular proliferation is attenuated.

Chapter 5 (Supplementary study): Regulation of GSTP1 by p53

Introduction

In light of recent data describing a p53 regulatory motif in the human *GSTP1* gene, the induction of GSTP1 by p53 was examined in addition to analysing transcriptional responses in response to GSTP1 silencing. As p53 is a prominent transcription factor in mediating cellular homeostasis, analysis of the regulatory mechanism between p53 and GSTP1 may lead to a better understanding of how GSTP1 can affect cell function independent of drug metabolism. This subchapter aims to address the role of p53 in mediating GSTP1 expression and later discusses a number of hypotheses with regards to the relationship between p53 and GSTP1.

5.5 p53

The p53 tumour suppressor is a transcription factor that is involved in the regulation of a number of cellular processes and maintenance of genome stability, often regarded as the 'guardian of the genome' (Lane, 1992). Normally p53 is tightly regulated through its degradation by MDM2, an E3 ubiquitin ligase (Haupt et al., 1997). Binding of p53 to MDM2 in the nucleus inhibits its transcription function and results in its export to the cytoplasm where it can be ubiquitylated (Wu et al., 1993, Ito et al., 2002). MDM2 itself is positively regulated by p53 and thereby creating a negative feedback for its own degradation. p53 can become stabilised and activated under a number of stress response pathways such as DNA damage (Shieh et al., 1997), heat shock (Wang and Chen, 2003), hypoxia (An et al., 1998), mitochondrial respiration (Matoba et al., 2006) and through the inactivation of tumour suppressors such as the retinoblastoma protein (Haupt et al., 1995). Typically, p53 is

stabilised through the phosphorylation of a number of serine residues (Siliciano et al., 1997, Cox and Meek, 2010) but is also subject to other post-translational modifications such as acetylation (Gu and Roeder, 1997). Stabilisation of p53 results in the initiation of intrinsic stress response pathways, resulting in the activation of a large number of downstream targets related to cellular arrest and apoptosis such as p21 (el-Deiry et al., 1994) and PUMA (Nakano and Vousden, 2001), as well as the recruitment of transcription factors such as p300/CBP (Avantaggiati et al., 1997). As such, p53 can be regarded as one of the principal transcription factors in responding to intrinsic cellular stress.

p53 is mutated in 50-70% of all human cancers, with mutations often leading to inactivation of apoptotic pathways and activation of novel gain of function mutations. Similar to GSTP1, the role of p53 in human tumours is complex and is involved in multiple cellular pathways related to proliferation and apoptosis. A number of studies have examined the potential link between p53 and GSTP1 in a number of cancers (Huang et al., 2009, Schumaker et al., 2008, Nakanishi et al., 1999) but no mechanistic link has been proposed. In examining a possible relationship between these 2 proteins, Gate et al crossed *Gstp1/2*$^{-/-}$ mice onto mice on a p53 null background (*p53*$^{-/-}$) and demonstrated that GSTP1 did not affect the tumour frequency on *p53*$^{-/-}$ mice, although, as described previously, GSTP1 did affect the tumour frequency in mice with wild-type p53 (Gate et al., 2005). It could also be hypothesised that regulation of JNK activation by GSTP1 may influence the transcription of p53 which is a downstream target of JNK (Fuchs et al., 1998). Activation of phosphorylated c-Jun forms part of the AP-1 complex resulting in the transcription of a number of stress response genes, including GSTP1 which contains an AP-1 site embedded into its antioxidant response element (ARE) in its promoter (Figure 1.3) (Xia et al., 1991, Moffat et al., 1994, Cowell et al., 1988). It is interesting to note that the human *GSTP1* promoter also contains a p53 regulatory motif in

intron 4 which has shown to be transcriptionally active (Lo et al., 2008) and therefore, like c-Jun, p53 may act in an auto-regulatory manner with GSTP1.

This supplementary chapter aims to briefly describe the transcriptional interplay between p53 and GSTP1 in light of recent findings demonstrating further p53 regulatory motifs in the *GSTP1* gene.

Results

5.6 Identification of further p53 regulatory motifs in the *GSTP1* gene

Bioinformatic analysis of the *GSTP1* gene confirmed the presence of a p53 regulatory motif in intron 4 (GGGCAAGCCT) of the human *GSTP1* gene as well as revealing a further p53 motif in the 3' non-coding region of the human *GSTP1* gene (AAACATGCTT), and putatively in exon 3 of the mouse *Gstp1* gene (AGGCTTGCTC). In order to determine whether GSTP1 was transcriptionally reliant on p53, the expression of GSTP1 was initially examined in p53 null HCT116 cells ($HCT116^{p53-/-}$, kindly provided by Dr. Bert Vogelstein). As demonstrated in Figure 5.7, Western blot analysis shows that deletion of p53 does not reduce the protein expression of GSTP1 in HCT116 cells. In contrast, deletion of p53 induces protein expression of GSTP1 and the expression of GSTM1. This phenotype is specifically mediated by the loss of p53 as deletion of p21, a downstream target of p53, does not have a similar effect. The increase in GSTP1 and GSTM1 protein expression correlates well with a decrease in HO-1 expression in $HCT116^{p53-/-}$ cells, indicative of a reduced oxidative stress response. This effect may be mediated by p53 acting as a suppressor of Nrf2-dependent transcription of antioxidant response genes (Faraonio et al., 2006) and therefore a lack of p53 may enhance the transcription and expression of genes such as GSTP1 and GSTM1, leading

to a decrease in cellular oxidative stress, evident by the decrease in HO-1 expression. Further evidence to suggest that p53 does not induce the transcription of GSTP1 is demonstrated in that transfection of p53 into HCT116$^{p53-/-}$ cells does not increase the expression of GSTP1. HCT116$^{p53-/-}$ cells were transfected with p53 (kindly provided by Dr. David Meek, University of Dundee) and GSTP1 expression was analysed 24 hours later. Despite our earlier observations in HCT116$^{p53-/-}$ cells, we do not observe a repression in GSTP1 levels 24 hours after transfection which suggests that the inhibition of GSTP1 expression by p53 may not be fully conclusive (Figure 5.8). However there is a notable shift in the migration of p53 on the Western blot, possibly as a result of a polymorphism and therefore the transfected p53, although wild-type, may not be endogenous to HCT116 cells. It has been demonstrated that an Arg72Pro substitution of p53, common within the human population, may account for differences in transcription (Marin et al., 2000) and therefore may account for the lack of GSTP1 response in this study.

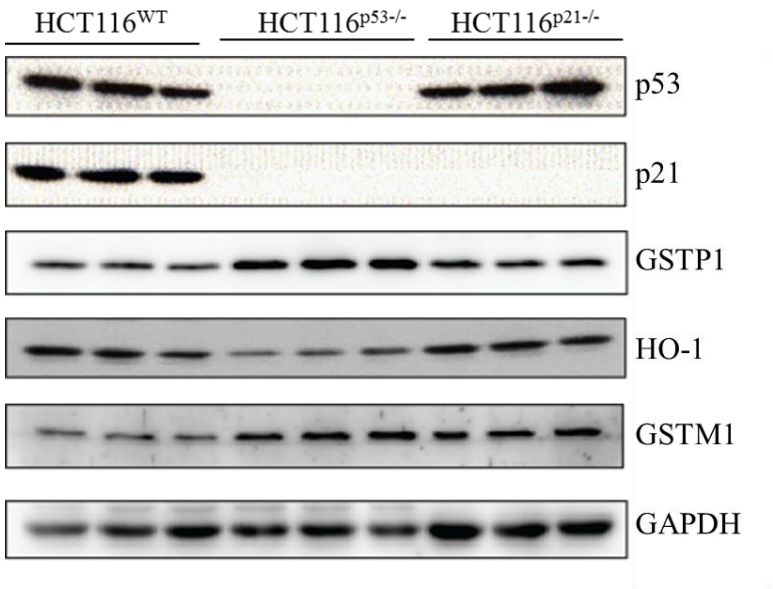


Figure 5.7. Deletion of p53 in HCT116 cells

HCT116, HCT116$^{p53-/-}$ and HCT116$^{p21-/-}$ cells were analysed for the basal expression of GSTP1. Cell lysates (10µg) were resolved on a SDS-PAGE gel and analysed by Western blotting. The blots demonstrate an induction in GSTP1 and GSTM1 expression in HCT116$^{p53-/-}$ cells, correlating with a decrease in HO-1 expression.

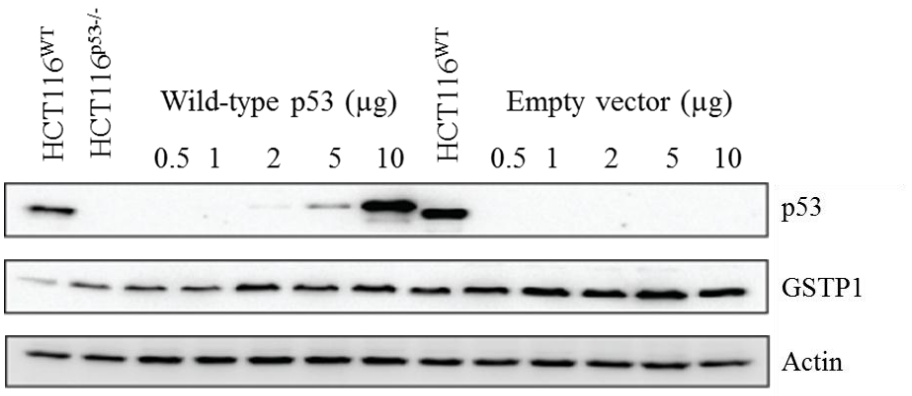


Figure 5.8. Transfection of wild-type p53 does not induce levels of GSTP1 in HCT116 cells.

HCT116$^{p53-/-}$ cells were transfected with increasing concentrations of p53 and analysed for GSTP1 expression 24 hours later. Cell lysates (10µg) were resolved on a SDS-PAGE gel and analysed by Western blotting.

5.7 Stabilisation of p53 does not alter the expression of GSTP1 in HCT116 cells

So far the data has examined basal expression of GSTP1 in response to deletion of p53, whilst p53-dependent activation of GSTP1 may only be mediated through induction of p53 in response to stress. Therefore the expression of GSTP1 was examined in response to a number of compounds which stabilise levels of p53 in HCT116 cells. $HCT116^{WT}$ and $HCT116^{p53-/-}$ cells were incubated with actinomycin D, cisplatin or etoposide for 8 hours and the expression of p53 and GSTP1 examined by Western blotting (Figure 5.9). As expected, each treatment stabilises the expression of p53 in $HCT116^{WT}$ cells but not in $HCT116^{p53-/-}$ cells. However, p53 stability does not correlate with activation of GSTP1. This experiment was also performed after 24 hours compound incubation and still no difference could be observed in the level of GSTP1 expression (data not shown). A notable increase in basal GSTP1 expression can be observed in $HCT116^{p53-/-}$ cells, correlating with our previous findings. Interestingly, a notable increase in NQO1 expression can be observed in $HCT116^{p53-/-}$ cells, which may provide further evidence for the induction of an antioxidant stress response in the absence of p53.

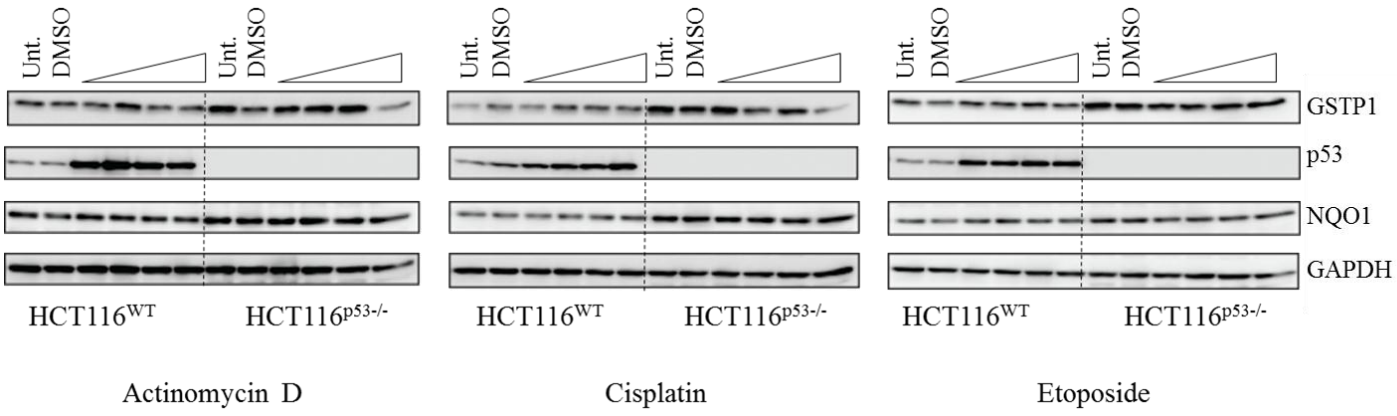


Figure 5.9. Stabilisation of p53 does not induce GSTP1 expression in HCT116 cells.

HCT116 and HCT116$^{p53-/-}$ cells were incubated with a number of p53 stabilising agents and the level of p53 and GSTP1 expression determined by Western blot analysis. Cells were incubated with Actinomycin D (25, 50, 100nM), cisplatin (10, 20, 40µM) or etoposide (25, 50, 100µM) for 8 hours before cell lysates were prepared and resolved (10µg) on a SDS-PAGE gel and analysed by Western blotting.

Finally, examination of p53 stabilisation on the activation or repression of GSTP1 was determined using a tetracycline-controlled (Tet-On) inducible p53 cell system (SAOS2/p53). SAOS2 cells are an osteosarcoma cell line that are null for p53 but have been transformed with a Tet-On vector which expresses p53 in the presence of doxorubicin (cells kindly provided by Dr. David Meek, University of Dundee). As demonstrated in Figure 5.10, treatment of SAO2 cells with doxorubicin induces the level of p53 specifically in SAOS2 cells transfected with the Tet-On system. However, induction of p53 does not lead to the activation of GSTP1 after 24 hours of doxorubicin treatment. Although the levels of GSTP1 appear to be suppressed in the presence of doxorubicin, the resting level of GSTP1 is markedly reduced in SAOS2/p53 cells which suggest that there may be some leakage of the Tet-On system. SAOS2 cells were grown in the presence of Tet-free FBS media and therefore it is unlikely that the culture media was responsible for any possible leakage. It is

also worth noting that SAOS2 cells lack a functional retinoblastoma protein (Rb) and therefore cannot sequester the E2F1 transcription factor which mediates cell proliferation and apoptosis in a p53 dependent and independent manner (Shaw and Tegtmeyer, 1981). Therefore, this may mask any apparent effects mediated by p53 in SAOS2 cells.

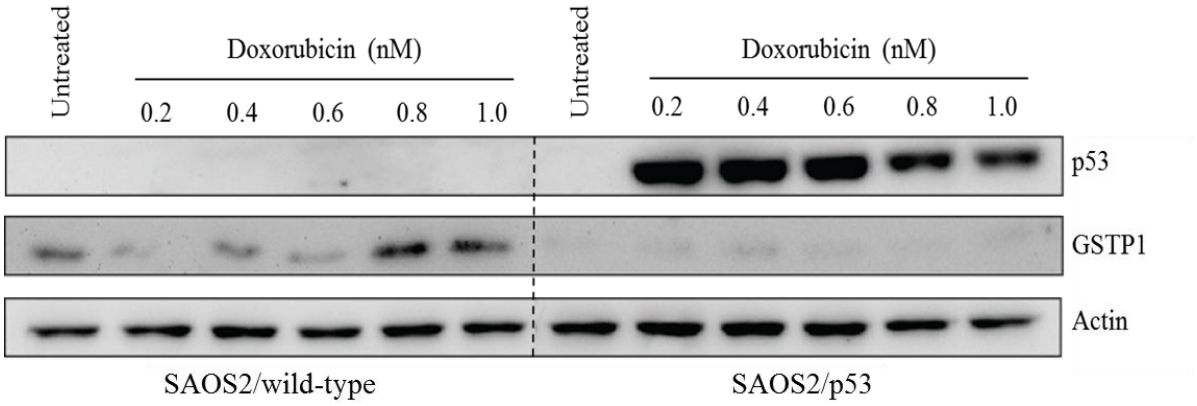


Figure 5.10. p53 induction in a p53-tet inducible SAOS2 cell line does not induce expression of GSTP1.

SAOS2/p53 cells contain an tet-inducible p53 expression vector which increases p53 expression in the presence of doxorubicin. SAOS2 wild-type or SAOS2/p53 cells were incubated with increasing concentrations of doxorubicin for 24 hours before cell lysates were prepared and resolved (10µg) on a SDS-PAGE gel and analysed by Western blotting.

In summary, this brief chapter highlights that despite the identification of further p53 regulatory motifs in the human *GSTP1* gene, a crude analysis of their regulatory function suggests that GSTP1 is not transcriptionally active in response to p53. Although these data are in contrast to those of Lo et al, they support the observations made by Faraonio et al, in that p53 may act to suppress the antioxidant response pathway (Faraonio et al., 2006). However, further work is required in order to examine this precise mechanism in relation to GSTP1 function.

6. Discussion

The data presented in this thesis begins to describe, for the first time, the non-catalytic functions of GSTP1 in an *in vivo* model and determine its role in defining resistance to a well-characterised hepatotoxin, acetaminophen (APAP). The results generated define novel mechanisms by which GSTP1 can mediate cell regulation whilst providing contradictory evidence to some mechanisms suggested in the literature. This discussion aims to corroborate the findings against the scientific literature whilst providing an outline of how GSTP1 is involved in mediating cell regulation.

6.1 Phenotype of *Gstp1*^{Y7F} mice and role of GSTP1 in mediating acetaminophen toxicity

It is clear from the data generated that the *Gstp1*^{Y7F} mouse harbours a GSTP1 protein devoid of catalytic activity. Targeting of the Tyr7 residue *in vivo* yields a GSTP1 protein which is transcribed and expressed to the same extent as its wild-type counterpart but is essentially catalytically inactive. *Gstp1*^{Y7F} mice show no activity towards ethacrynic acid whilst recombinant protein studies demonstrate that the Y7F protein can still bind GSH and CDNB. There were no compensatory changes in other GSTs within the liver and any basal phenotypic differences as determined by organ weight appear to be sex-related rather than genetic; the reasons for this at present are unexplained. Basal ratios and concentrations of GSH:GSSG did not differ between *Gstp1*^{Y7F} or *Gstp1*^{WT} mice and no change was evident when compared to *Gstp1/2*$^{-/-}$mice. These observations suggest that GSTP1 is not critical in murine development or in the regulation of basal redox control. These results support previous findings associated with the deletion of GSTP1 from mice at resting levels, although

some subtle differences are apparent. These are mostly related to mouse strain, such as differences in lung size in *Gstp1/2*$^{-/-}$ mice generated on a 129xMF1 background (Henderson et al., 1998a), which were not evident on a C57Bl/6J background.

In vivo studies of GSTP1 have previously demonstrated that this enzyme forms part of an integrated adaptive response in maintaining cellular homeostasis following chemical and genetic stress. Unlike most forms of insult, the presence of GSTP1 appears to potentiate the hepatotoxicity of APAP in mice and therefore we investigated whether the catalytic activity of GSTP1 is responsible for this phenotype. The use of a non-catalytic GSTP1 mouse model revealed that the hepatotoxicity associated with APAP is mediated through the catalytic activity of GSTP1, as APAP treatment of *Gstp1*^{Y7F} mice does not cause serum transaminase induction or hepatic centrilobular necrosis. As mice nulled for GSTP1 share a similar phenotype with mice harbouring a Y7F mutation, it is unlikely that a non-catalytic GSTP1 has acquired novel protective functions in the protection of APAP induced toxicity. Therefore the toxicity associated with APAP is most likely potentiated by the catalytic activity of GSTP1.

There are three stages in which the catalytic activity of GSTP1 may contribute to the hepatic necrosis associated with APAP administration. These are described at three distinct phases of NAPQI induced toxicity as shown in Figure 6.1. Firstly, GSTP1 may contribute to APAP-induced hepatic necrosis through the rapid depletion of the hepatic GSH pool, as shown in step 1 in Figure 6.1. In Figure 3.10A we observe a marked decrease in the rate of GSH depletion over that in *Gstp1/2*$^{-/-}$ or *Gstp1*^{Y7F} mice, correlating with a quicker increase in the production of glutathione disulphide formation after 40 minutes of APAP treatment (Figure

3.10B). The catalytic properties of GSTP1 may mediate the rapid conjugation of NAPQI to GSH, resulting in a faster depletion of hepatic GSH and lead to a quicker onset of subsequent mechanisms responsible for hepatic necrosis. The reduced rate of GSH depletion in other mouse lines may allow time for a comprehensive response in antioxidant and detoxification pathways which are not apparent in *Gstp1*^{WT} mice. However, it has been demonstrated using diethylmaleate that depletion of GSH itself is not sufficient to induce tissue damage (Mitchell et al., 1973) whilst others have shown wide variation of hepatocellular damage using a variety of GSH depleting agents (Kitteringham et al., 2000) and therefore further downstream mechanisms associated with NAPQI must account for the subsequent necrosis. This becomes apparent when examining the level of GSH depletion in *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice, as despite a slower rate of GSH depletion, there is no difference in the level of GSH after 90 minutes when compared to *Gstp1*^{WT} mice. As there does not appear to be any difference in the level of covalent binding of GSH to APAP or differences in cytochrome P450 2E1 between genotypes (Henderson et al., 2000), GSTP1 mediated conjugation of NAPQI does not appear to be a likely mechanism by which GSTP1 potentiates hepatic necrosis.

The second stage centres on the function of GSTP1 when GSH levels are minimal (point 2, Figure 6.1). At this time point, there are very few differences in phenotype across all genotypes as determined by MAP Kinase activation, GSSG levels and oxidative stress. Despite this, there are clear differences in these parameters, with the exception of oxidative stress, subsequent to GSH depletion. This, therefore, appears to be the key time point at which APAP resistance is mediated in the absence of a catalytically functional GSTP1. Despite few compensatory changes in hepatic protein expression at resting levels in *Gstp1/2*$^{-/-}$ mice (Kitteringham et al., 2003), APAP-induced toxicity may elicit a stronger response in compensatory changes, particularly at a time point when GSH depletion is maximal and

oxidative stress is high. Future experiments would include proteomic analysis of mouse liver at the point of maximal GSH depletion. This would be complemented with the use of microarray profiling at this time point, as 40-90 minutes post-APAP dosing may be too early a time point to identify changes in protein expression, but would provide a detailed transcriptional response to APAP as well as an understanding of the biological processes taking place. Although the covalent binding of NAPQI does not differ between *Gstp1*^{WT} and *Gstp1/2*$^{-/-}$ mice at non-toxic doses, it would be interesting to determine if the distribution of this metabolite is a factor in mediating differences to APAP toxicity through the use of radiolabelled APAP.

The final stage involves the regulation of cellular pathways after GSH pools have been depleted (highlighted at point 3 in Figure 6.1). There are clear biochemical differences in a number of assays measured between *Gstp1*^{WT} and *Gstp1/2*$^{-/-}$*/Gstp1*^{Y7F} mice 240 minutes after APAP dosing, including the activation of MAP Kinase pathways (Figure 3.11) and levels of hepatic GSH (Figure 3.10A). As the maximal level of GSH depletion is similar across all genotypes, it could be reasoned that functions of a catalytic GSTP1 contributing to cellular necrosis occur only after maximal depletion of GSH. Whether or not the mechanism by which GSTP1 potentiates this effect is a continuation of those discussed in the second stage (point 2, Figure 6.1) or is a separate function subsequent to GSH depletion is unclear. It would also be interesting to determine if the mechanism underlying the resistance mediated in *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice is sustained throughout the entire time course or whether an acute functional response after GSH depletion is necessary to avoid toxicity.

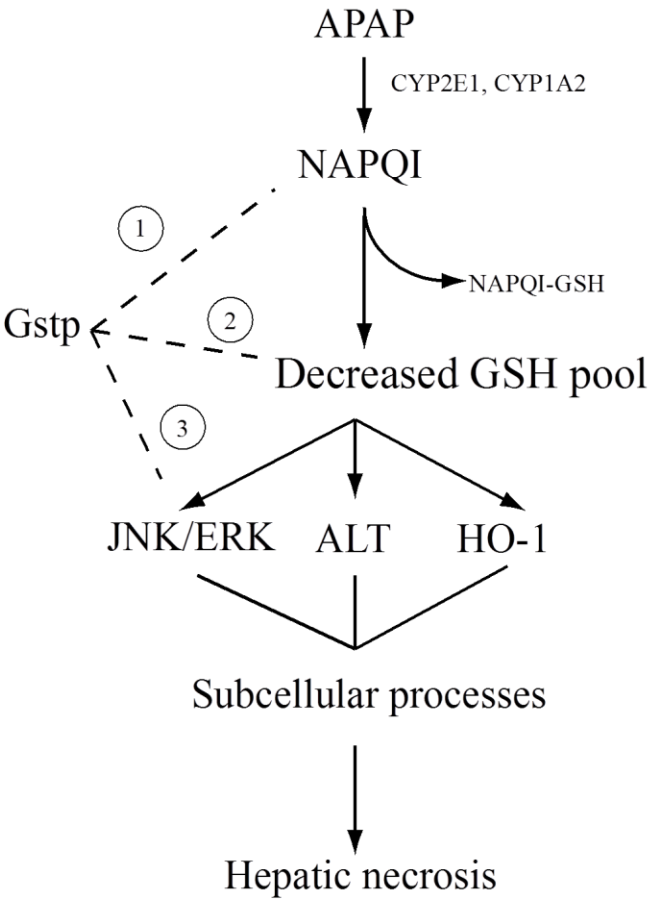


Figure 6.1. Schematic figure outlining the route to hepatic necrosis from acetaminophen (APAP) overdose in mice.

From the data generated in Chapter 3, APAP administration to mice (300mg/kg, oral dose) induces hepatic necrosis through a series of mechanistic pathways involving the catalytic activity of GSTP1. The precise mechanism in which the catalytic activity of GSTP1 mediates these effects is not yet determined but 3 hypotheses have been suggested. There are numbered in the diagram and outlined in the text.

6.2 Mechanisms of GSTP1 mediated acetaminophen induced toxicity

6.2.1 Oxidative stress and inflammation

The stages highlighted in Figure 6.1, although alluding to the process of APAP-induced toxicity and the kinetics of GSTP1 association with APAP, do not provide an accurate account of the mechanism by which GSTP1 can potentiate APAP toxicity. It is therefore important to assess the likely mechanisms surrounding APAP toxicity and discuss the wider implications of GSTP1-mediated cell regulation in light of the data presented in this thesis. In contrast to many previous publications, the mechanism behind APAP toxicity appears to be independent of oxidative stress. There is no apparent difference in the levels of glutathione disulphide or HO-1 after 90 minutes APAP administration, the time points at which we begin to observe GSH regeneration and silencing of MAP Kinase pathway in *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice (Figure 3.10B and Figure 3.11). After 24 hours of APAP treatment, HO-1 expression could be observed surrounding the centrilobular regions in *Gstp1*^{Y7F} and *Gstp1/2*$^{-/-}$ mice and therefore the level of oxidative stress is not indicative of the severity of toxicity. This finding supports unpublished data from *Gstp1/2*$^{-/-}$ mice crossed onto mice carrying a HO-1 reporter transgene treated with APAP (Vasey, Henderson, unpublished). *LacZ* is fused to the C-terminus of HO-1, induction of which results in the expression of both HO-1 and *lacZ*, which can be identified using ß-galactosidase staining. APAP treatment of these mice demonstrates ß-galactosidase staining around the centrilobular regions of *Gstp1/2*$^{-/-}$ mice and around the 'necrotic rings' in *Gstp1*^{WT} mice (Figure 6.2), which is in agreement with our observations of HO-1 induction by APAP.

There are a number of studies highlighting an inflammatory response to APAP treatment which demonstrate an up-regulation of a number of pro-inflammatory markers and pathways

such as TNFα (Blazka et al., 1995b) and IFNγ (Ishida et al., 2002). However, there are some contradictions to these observations (Boess et al., 1998), highlighting the complex nature of inflammation in APAP induced toxicity. The observation that GSTP1 can potentiate myeloproliferation in mice generated on a mixed background may provide an insight into potential inflammatory response which may account for these observations (Gate et al., 2004). However, data generated but not published in this thesis show that, in C57/Bl6J mice, there is no difference in the level of myeloproliferation between *Gstp1*^{WT} and *Gstp1/2*$^{-/-}$ mice, and therefore is unlikely to account for differences in APAP toxicity. Additionally, there are a number of studies which suggest that inflammation is suppressed in *Gstp1*^{WT} mice compared to *Gstp1/2*$^{-/-}$ mice (Ritchie et al., 2009, Henderson et al., 2011) and therefore the mechanism behind APAP resistance in *Gstp1/2*$^{-/-}$ mice is unlikely to be related to inflammation.

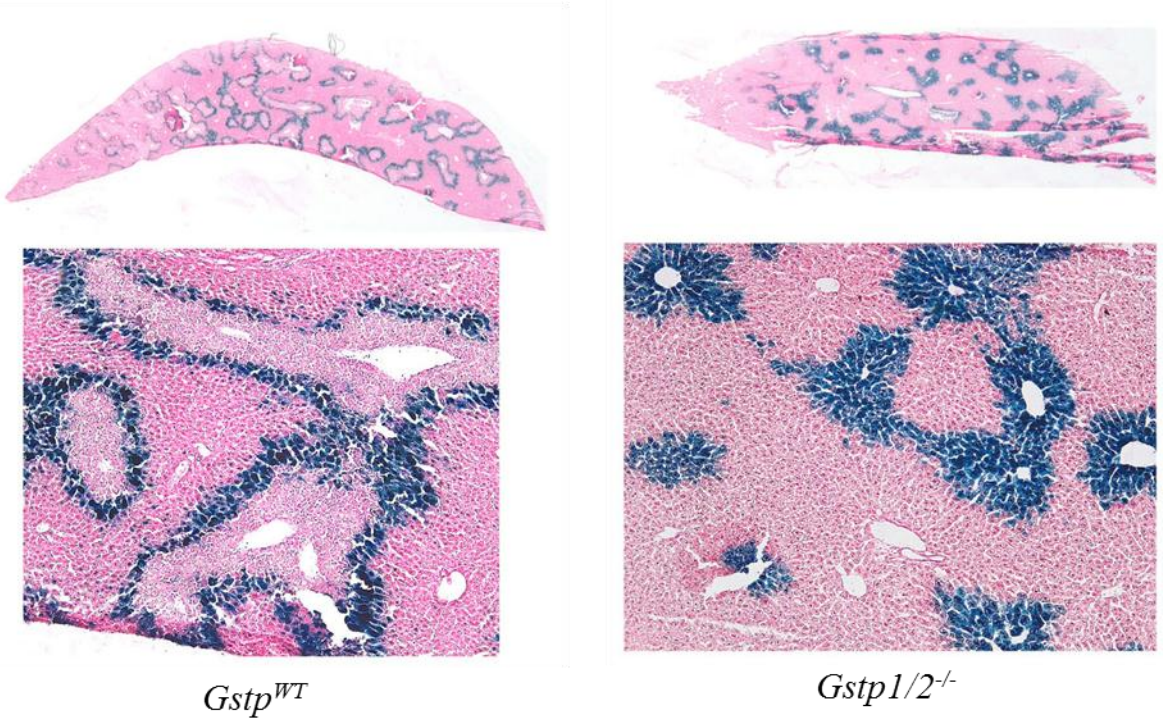


Figure 6.2. APAP treatment induces HO-1 expression in *Gstp1*^{WT} and *Gstp1/2*$^{-/-}$ mice

Gstp1^{WT} and *Gstp1/2*$^{-/-}$ mice crossed onto mice harbouring a HO-1 *lacZ* reporter transgene were administered a single oral dose of 300mg/kg APAP and harvested 24 hours later. Livers were removed and frozen before cut into 15µm sections and stained using ß-galactosidase to determine the expression of HO-1. Data kindly provided by Dr. Douglas Vasey and Dr. Colin Henderson.

6.2.2 GSTP1 mediated MAP Kinase regulation

Similarly, the role of GSTP1 in inhibiting JNK activity does not appear to account for the differences in APAP toxicity. APAP treatment in mice has been shown to increase AP-1 binding to DNA through the increased phosphorylation of c-Jun (Kitteringham et al., 2000) and is up-regulated during tissue necrosis but not hepatic inflammation (Blazka et al., 1996) leading to the release of inflammatory mediators (Blazka et al., 1995a). Despite a number of studies highlighting the role of GSTP1 in inhibiting the phosphorylation of c-Jun through JNK inhibition, no propagation of JNK activity could be observed in *Gstp1/2*$^{-/-}$ mice

compared to *Gstp1*^{WT} mice after APAP administration and therefore does not adhere to the conventional theory that deletion of GSTP1 leads to increased JNK activation. However, the noticeable increase in JNK phosphorylation in *Gstp1*^{WT} mice agrees with many published data, highlighted in Chapter 3, that JNK activation is required for tissue necrosis in response to APAP treatment. The observation that ERK activation is also increased in *Gstp1*^{WT} mice, suggests a general activation of the MAP Kinase pathway, independent of specific GSTP1 regulation of JNK. It is also interesting that there is little difference in the level of JNK/ERK activation between *Gstp1*^{WT} and *Gstp1*^{Y7F} mice, despite a large difference in the sensitivity to APAP. Firstly, this highlights that the catalytic function of GSTP1 is not necessary in mediating signalling through MAP Kinase cascade. This is supported through a study by Adler et al who established that GSTP1 binding of JNK is established on residues other than Tyr7 (Adler and Pincus, 2004). It is also interesting that the localisation of ERK activation is similar in *Gstp1*^{WT} and *Gstp1*^{Y7F} mice i.e., centrilobular. This, along with the observation that HO-1 expression localises to similar regions, highlights an interesting observation in the specificity of the hepatic expression of these genes. Secondly, it also suggests that JNK/ERK activation may not be the dependent factors in establishing hepatic necrosis. There are a number of studies which show a decrease in NF-κB binding associated with APAP toxicity (Chia et al., 2010, Blazka et al., 1995a) suggesting that a multi-transcriptional response is responsible for the development of tissue necrosis in response to APAP. Therefore, despite their similar phenotype in mediating cell regulation, the catalytic function of GSTP1 is contributing to the toxicities associated with APAP independently of MAP Kinase regulation.

Further evidence to support mechanisms of cell regulation other than JNK regulation by GSTP1 is demonstrated in HCT116 cells silenced for GSTP1, as evident in Chapter 4. Silencing of GSTP1 did not induce changes in the level of JNK phosphorylation or that of c-

Jun, even after UV treatment, suggesting that GSTP1 must have cellular functions other than JNK inhibition. Adler et al demonstrated that the amino acid residues Ile105 and Ala114, mutations of which confer the different polymorphisms of GSTP1, are crucial for binding of GSTP1 to JNK (Adler and Pincus, 2004), while Holley et al demonstrated different levels of JNK activity between the 2 haplotypes *GSTP1*A* and *GSTP1*C* (Holley et al., 2007). Recent data also suggest that GSTP1 mediates its regulation of JNK through an interaction with activating transcription factor 2 (ATF2) (Thevenin et al., 2011). Differences in the affinity of the *GSTP1*B* polymorphism, identified in HCT116 cells, for JNK or ATF2 may explain a lack of JNK regulation mediated by GSTP1 in this cell line. Microarray analysis of cells stably silenced for GSTP1 did not show any significant changes in the expression of transcription targets downstream of JNK, such as c-Fos, c-Jun and ATF2, correlating with a lack of JNK mediated signalling. However, it is interesting to note that HCT116 cells are heterozygous for a mutation in *KRAS* (Brink et al., 2003), activation of which has been shown to regulate members of the MAP Kinase pathway (Dunn et al., 2011, Weinberg et al., 2010) and therefore it is feasible that *KRAS* activation could ameliorate any relationship between GSTP1 and JNK due to sustained oncogenic signalling.

6.2.3 Novel functions of GSTP1 mediated signalling

The recent identification of two novel functions of GSTP1 may provide a clear insight into APAP induced hepatotoxicity. Firstly, there are numerous studies relating acetaminophen induced toxicity with a deregulation in the chelation of iron and nitric oxide production. The reduction of peroxides by ferrous irons occurs via a Fenton mechanism, leading to the production of radical species which increase with APAP toxicity and interact with a number of lipids and proteins. The addition of an iron chelator to cultured hepatocytes prevents acetaminophen toxicity (Kyle et al., 1987) and it has been shown that incubation with the iron

donor 3,5,5-trimethyl-hexanoyl ferrocene increases APAP associated toxicities without inducing basal changes in ROS, mitochondrial function or oxidative stress (Moon et al., 2010). A recent study by Lok et al has shown that GSTP1 binds to dintrisosyl-iron complexes (DNICs) in regulating intracellular levels of nitric oxide and iron release, preventing their efflux from cells via the multidrug resistance protein 1 (Lok et al., 2012) whilst another study by Cesareo et al demonstrated that Tyr7 was the key residue in targeting the iron atom (Cesareo et al., 2005). It could be hypothesised that the prevention of DNIC efflux in hepatocytes, as a result of GSTP1 sequestering, leads to the accumulation of iron and nitric oxide within the cell, which in turn contributes to hepatic necrosis through the generation of peroxide radicals such as peroxynitrite. In *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice, DNICs are not sequestered by GSTP1 in the cell and therefore DNIC accumulation is attenuated. There are some concerns with this model as it has been proposed that DNIC regulation by GSTP1 acts to suppress the toxicities associated with DNIC as a NO carrier and has been demonstrated to increase the resistance of cells against GSNO in collaboration with MRP1 (Lok et al., 2012). Increased efflux of DNIC in *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice may lead to increased extracellular nitric oxide release which could potentially lead to inflammation and toxicity, which are not observed in these mice after APAP treatment. It has been observed that inhibition of GSH prevents NO-mediated iron release through the DNIC complex (Watts and Richardson, 2001) and therefore any level of discrepancy between GSTP1-mediated sequestering of NO may be ameliorated when GSH levels are depleted due to APAP treatment. DNICs have also been shown to inhibit the activity of glutathione reductase via nitrosation (Boese et al., 1997) while the presence of GSTs protects glutathione reductase and restores its activity (Pedersen et al., 2007). Therefore one would expect differences in the ratio of GSH:GSSG if glutathione reductase is unable to reduce glutathione disulphide. This may be evident 40 minutes after APAP treatment (Figure 3.10B) but the difference is soon lost 90 minutes after APAP

treatment and therefore unlikely that the role of GSTP1 in mediating DNIC efflux is a coherent mechanism by which APAP sensitivity is propagated *in vivo*.

The second mechanism of GSTP1 function related to APAP-induced toxicity examines its role in protein S-glutathionylation and GSH homeostasis. The inability of *Gstp1*^{WT} mice to regenerate hepatic GSH levels after APAP treatment appears to be the predominant factor contributing to hepatic necrosis. Increased hepatocyte death/injury after treatment with APAP would lead to a reduction in the number of viable cells able to synthesise GSH which may account for the inability of *Gstp1*^{WT} mice to regenerate hepatic GSH. The lack of hepatic injury in *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice enables hepatocytes to regenerate GSH although the precise mechanism by which these mice develop APAP resistance remains unclear. Our *in vitro* data from HCT116 cells suggests that GSTP1 appears to play a role in GSH homeostasis as transient or stable silencing of GSTP1 results in increased glutathione disulphide formation. Despite a lack of change in redox state basally *in vivo,* it is clear that GSTP1 plays a role in redox regulation, particularly in response to stress. As a colon carcinoma cell line, HCT116 cells possess a number of activated oncogenic and stress-related signalling pathways which are not activated basally *in vivo* and therefore may account for the differences in redox state between models. A key experiment to assess the role of GSTP1 in the glutathione homeostasis would be to treat *Gstp1*^{WT}, *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mice with a GSH-depleting agent which does not induce hepatic toxicity and evaluate the rate of GSH regeneration. In this way, the role of GSTP1 is specifically controlled for the regulation of GSH homeostasis and not in the detoxification of a hepatotoxin. In *Gstp1*^{WT} mice, a single i.p. dose of BSO (4mmol/kg) results in 60% depletion of hepatic GSH levels over a 4 hour period and fully regenerates after 24 hours without inducing changes in serum transaminase levels (data not shown). If the function of GSTP1 is intrinsically related to GSH homeostasis

and independent of hepatic toxicity, we should observe a quicker regeneration of hepatic GSH in the absence of a catalytically functioning GSTP1.

As described previously in Chapter 4, GSTP1 has been shown to catalyse global protein S-glutathionylation in response to oxidative and nitrosative stress and is dependent on its Tyr7 residue to mediate this effect (Townsend et al., 2008a). Based on this finding alone, one would hypothesise that more proteins would be S-glutathionylated in *Gstp1*^{WT} mouse hepatocytes in response to APAP treatment than in *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F} mouse hepatocytes. S-glutathionylation is thought to be a protective mechanism against oxidative stress while the majority of S-glutathionylation reactions lead to reversible inhibition of protein activity. Therefore it is conceivable that if a protein critical in the regeneration of GSH synthesis, for example GCL, is S-glutathionylated, its catalytic activity may be inhibited and glutathione regeneration cannot occur. However, Yang et al demonstrated that S-glutathionylation of cellular proteins decreases in areas of hepatic damage induced by APAP, with unaffected areas exhibiting high levels of protein S-glutathionylation (Yang et al., 2012). In Chapter 4, it was demonstrated that transient knockdown of GSTP1 in HCT116 cells, induced basal levels of protein S-glutathionylation. From these data, one could hypothesise that *Gstp1/2*$^{-/-}$ and *Gstp1*^{Y7F}mice retain high levels of protein S-glutathionylation in response to APAP treatment resulting in the protection of protein thiols from labile *ipso* NAPQI adduct formation. If the proposed GSTP1:DNIC model (see earlier) is correct, then *in vivo* nitric oxide unscavenged by GSTP1 may, instead of causing toxicity, act as an intermediate for S-glutathionylation through the S-nitrosylation of cellular proteins. Generation of mouse hepatocytes would provide an incredibly powerful tool in examining this potential mechanism by which GSTP1 mediates sensitivity to APAP, as the *in vitro* methods used to detect protein S-glutathionylation outlined in Chapter 4 could be applied.

BioGEE incubation of hepatocytes and subsequent mass spectrometric analysis would enable the identification of proteins specifically S-glutathionylated after APAP treatment. Not only would this further our understanding of the role of GSTP1 in mediating S-glutathionylation in response to stress, but may also provide a 'footprint' of proteins which are subject to S-glutathionylation in mediating a response to APAP treatment as very few *in vivo* methods of examining S-glutathionylation have resulted in the identification of proteins modified in such a manner.

6.3 GSTP1 does not act as a catalyst in protein S-glutathionylation in HCT116 cells

The data presented here are in contrast to those published by Townsend et al (Townsend et al., 2008a), on the role of GSTP1 in the S-glutathionylation of cellular proteins as transient silencing of GSTP1 increased basal levels of protein S-glutathionylation in HCT116 cells. This is possibly due to the induction of glutathione disulphide formation within the cell, leading to increased protein S-glutathionylation through disulphide exchange. The observation that HCT116 cells stably silenced for GSTP1 showed increased glutathione disulphide formation but no change in protein S-glutathionylation suggests that this mechanism may not be universal. However, the observation that glutathione disulphide levels are increased in both transient and stable cell lines demonstrates that GSTP1 has a functional role in glutathione homeostasis *in vitro*. Despite a reduction in protein S-glutathionylation in the presence of an iNOS inhibitor, some recent data suggest that there may be a role for nitrosylated proteins to act as a precursor to S-glutathionylation in this context. Using a biotin-switch protocol, in which nitrosylated bonds are reduced using ascorbate and subsequently labelled with a thiol-specific biotin reagent (Jaffrey and Snyder, 2001, Forrester et al., 2009), we can show that there is an increase in the amount of nitrosylated proteins in HCT116 cells transiently silenced for GSTP1 (Appendices VI) although further work is

required to verify this. Another possibility is that a reduction in the deglutathionylation of cellular proteins allows for the accumulation of S-glutathionylated proteins in response to GSTP1 silencing, such as the inhibition in the activity or transcription of glutaredoxin. However, the observation that glutaredoxin mRNA is upregulated in stable GSTP1 silenced HCT116 cells suggests that this may not be the case. It is possible that GSTP1 itself may act to reduce S-glutathionylation through the removal of thiol groups from proteins. As described in the Introduction, the N-terminal fold domain of GSTP1 contains a βαβαββα motif, similar to that of the thioredoxin family of enzymes, and therefore it is conceivable that GSTP1 may possess similar reduction properties which are conserved through evolution. However, it is unclear what the kinetics of such a reaction would be and what type of co-factors would contribute to the reaction. Similarly to the glutathione disulphide model, as no difference in S-glutathionylation was observed in stable knockdown cells such a mechanism may not be universal.

A more likely mechanism for the transient induction of S-glutathionylation is related to changes in the oxidation state of the cell. Treatment of BSO in HCT116 cells induced a 1.5 fold increase in ROS production in HCT116 cells silenced for GSTP1, leading to a large increase in protein S-glutathionylation. Although we could not detect any changes in NQO1 or HO-1 induction in GSTP1-silenced cells, protein S-glutathionylation is a transient response to prevent oxidation of cysteine groups and therefore it is feasible that the S-glutathionylation of cellular proteins occurs before a stress response signal is observed. Kinetically, this is favourable as less energy is required for the conjugation of thiol groups with a glutathionyl anion compared to the transcription and translation of new proteins. One can account for the observations of Townsend et al through this mechanism as PABA/NO, the compound used to induce nitrosative stress and S-glutathionylation in their study, is more

cytotoxic to *Gstp1*^{WT} MEFs than to *Gstp1/2*$^{-/-}$ MEFs (Townsend et al., 2006) and therefore the increase in protein S-glutathionylation may be as a result of increased cytotoxicity and not as a function of GSTP1. However, the fact that GSTP1 can facilitate the conjugation of GSH with other xenobiotic compounds suggests that it may facilitate the conjugation of GSH in certain conditions. If this is true, an interesting question is whether GSTP1 can solely facilitate this process or if this is a function of all cytosolic GST classes; if there are differences between GST classes, what accounts for these differences?

Protein S-glutathionylation in this thesis has shown to protect cells from the alkylating effects of N-ethylmaleimide and contributes further to the concept that S-glutathionylation plays a functional role in the protection of thiol groups. Interestingly, p53 has also been shown to be S-glutathionylated in the presence of oxidative stress, conferring a functional change *in vitro* which reduces its ability to bind to DNA (Velu et al., 2007). This observation has been supported *in vivo*, where S-glutathionylation of p53 in Alzheimer's disease is thought to prevent its formation as a tetramer due to the high level of oxidative species often associated with the disease (Di Domenico et al., 2009). The data provided in Sub-chapter 5, may allude to a cellular function of this mechanism. Despite identifying further p53 regulatory motifs in the 3' non-coding region of the human *GSTP1* gene, and putatively in exon 3 of the mouse *Gstp1* gene, we could not find any evidence in HCT116 or SAOS2 cells that the motifs are transcriptionally active. In the absence of p53, we observe an increase in a number of ARE-regulated genes which is agreeable with those observations made by Faraonio et al (Faraonio et al., 2006). One could hypothesise that in times of oxidative stress, S-glutathionylation results in the inactivation of p53, allowing for the activation (via Nrf2) of a number of antioxidant response genes in order to combat cellular stress, without inducing cellular arrest or apoptosis. If there are conditions in which S-glutathionylation is catalysed by GSTP1 or

indeed reduced, this may have serious implications associated with p53 cell regulation, particularly in cancer where GSTP1 is often over-expressed, or where pharmacological agents act to inhibit GSTP1 function.

6.4 GSTP1 as a potential regulator of the Hsp90 pathway

The hypothesis relating protein S-glutathionylation to p53 function was difficult to interpret in our *in vitro* culture system as stable knockdown of GSTP1 did not induce changes in global protein S-glutathionylation. Additionally, knockdown of GSTP1 did not appear to induce large differences in sensitivity to a number of cytotoxic compounds in HCT116 cells with only a few compounds increasing the sensitivity of $HCT116^{shGSTP1}$ cells. The increased sensitivity of these cells to the Hsp90 inhibitor 17-AAG is complex to describe, especially as a number of Hsp90 related pathways appear upregulated in $HCT116^{shGSTP1}$ cells and another Hsp90 inhibitor, NVP-AUY922, did not show any difference in cytotoxicity. However, a relationship between GSTP1 and Hsp90 may exist as a result of their regulation from a family of serine protease inhibitors known as Maspin. Maspin has been shown to upregulate the expression of GSTP1 through inhibition of histone deacetylase 1, resulting in demethylation of the *GSTP1* promoter (Li et al., 2011). Subsequent expression of GSTP1 confers resistance to oxidative stress induced by H_2O_2 as well as increases maspin-mediated HDAC1 inhibition. The Li group have also speculated that Maspin can inhibit HDAC1-mediated Hsp90 deacetylation as Maspin has been found to bind to Hsp90 (Yin et al., 2005) and therefore a possible link may exist whereby the extent of Hsp90 deacetylation is dependent on the expression of GSTP1. The use of 17-AAG clinically may be difficult to justify in tumours containing low expression of GSTP1 as a phase II trial of patients with metastatic, hormone-refractory prostate cancer, demonstrated that 17-AAG did not appear to

decrease the levels of prostate-specific antigen (PSA), a marker of prostate cancer (Heath et al., 2008).

6.5 GSTP1 localisation and function within the mitochondria

Previous data generated from cancer cell lines overexpressing recombinant GSTP1 suggests that GSTP1 confers resistance to a number of mitochondrial toxins by reducing the oxidative stress within the mitochondria (Goto et al., 2009) and therefore may play a role in the protection of hepatocytes against APAP toxicity. Although some evidence generated from the microarray profiling suggested that HCT116^{shGSTP1} cells had higher basal levels of oxidative stress, H_2O_2 treatment of these cells did not show any significant difference in cytotoxicity from control cells (data not shown). The data presented here demonstrates that GSTP1 can localise to the mitochondria, although its function is still unclear. Although there is a slight trend provided in Chapter 4 demonstrating decreased resistance to rotenone treatment in HCT116^{shGSTP1} cells, there is little significant data provided in this thesis to support the role of GSTP1 in mediating protection of the mitochondria against toxins. The function of other GST classes within the mitochondria, such as GST Kappa (Harris et al., 1991) and GST Alpha (Raza et al., 2002) may compensate for the loss of GSTP1, especially as its expression within the mitochondria is very low compared to other cellular compartments. Further data demonstrates that the presence of a catalytic GSTP1 protein does not appear to confer resistance to rotenone in MEFs, although interestingly, MEFs expressing GSTP1 or its non-catalytic derivative respire at a higher rate than compared to *Gstp1/2*$^{-/-}$ MEFs. Despite respiring at a lower basal rate, mitochondria from *Gstp1/2*$^{-/-}$ MEFs appear to be driven harder in the presence of a mitochondrial uncoupling agent, DNP, than mitochondria from *Gstp1*^{WT} or *Gstp1*^{Y7F} MEFs. The low level of glycolysis in *Gstp1/2*$^{-/-}$ MEFs, even after induction with DNP suggests that GSTP1 localisation within the mitochondria may aid in MEFs

preferentially undertaking glycolysis than oxidative phosphorylation, and that this effect can be mediated non-catalytically. This is a difficult concept to prove from the data generated, as we could not repeat this finding in HCT116 cells silenced for GSTP1. As the technology and interpretation for establishing mitochondrial respiration and glycolysis is relatively new, more optimisation is required using a number of mitochondrial toxins to examine the exact mitochondrial function of GSTP1. There is evidence from Yeast two-hybrid studies to suggest that GSTP1 can interact with a number of mitochondrial proteins, particularly ETFα; however we could not find any evidence for this through immunoprecipitation of GSTP1-tagged constructs. Further experiments utilising mass spectrometric profiling of immunoprecipitated proteins are required to establish the role of GSTP1 in the regulation of cellular proteins. This would be performed under a number of different cellular stressors such as mitochondrial, oxidative, ER and DNA damaging agents so as to establish how GSTP1 regulates cell function in response to stress.

6.6 Proliferation of HCT116 cells is mediated by GSTP1

GSTP1 has previously been demonstrated to be an important factor in cellular proliferation. Transient or stable silencing of GSTP1 reduces the growth rate of HCT116 cells, and the mechanism by which this occurs is independent of JNK signalling. This function appears to be specifically mediated in tumour cells as primary *Gstp1/2*$^{-/-}$ MEFs, in contrast, show a higher proliferation rate than their wild-type counterparts (Ruscoe et al., 2001). Therefore it is likely that growth signalling mediated by GSTP1 in tumour cells is a secondary mechanism to a change in cellular function in the development of cancer. The finding of a down-regulation in MKNK2 expression in HCT116^{shGSTP1} cells may account for the reduced proliferation and a down-regulation of a large number of pathways related to cytoskeleton formation. Down-regulation of MKNK2 leads to a reduction of eIF4E phosphorylation

resulting in reduced mRNA translation and cell proliferation (Ueda et al., 2010). The microarray also identified Regulator of G-protein Signalling 2 (RGS2) as up-regulated in $HCT116^{shGSTP1}$ cells. RGS proteins negatively regulate G-protein-coupled receptor signalling by binding to active G protein alpha subunits leading to reduced signal transduction (Chen et al., 2000a, Heximer et al., 1997). RGS2 has been shown to inhibit glucose-induced cAMP signalling (Tseng and Zhang, 1998) and regulates T-cell proliferation and IL-2 production (Oliveira-Dos-Santos et al., 2000). While studies show that RGS2 is overexpressed in certain types of cancer (Boelte et al., 2011) RGS2 mRNA expression has been found down-regulated in colon cancer and associated with poor survival (Jiang et al., 2010), highlighting a potential relationship between GSTP1 expression and the expression of RGS2. Together, these data suggest that GSTP1 plays a key role in the proliferation of cancer cells through the regulation of a number of proteins involved in cell growth, although the nature of the relationship between these proteins remains to be determined.

7. Conclusions

The data presented in this thesis clearly demonstrate that the function(s) of GSTP1 extends well beyond enzymology and is independent of its catalytic activity. There are many examples from the literature of how GSTP1 can mediate cell function and it is clear from this thesis that the functions of GSTP1, other than drug metabolism, appear to be context- and stress-dependent. The development of an *in vivo* model to demonstrate the non-catalytic role of GSTP1 is of great importance in understanding how this enzyme regulates cellular processes. One of the strengths of the data presented is that, for the first time, a clear relationship has been demonstrated *in vivo* between the catalytic activity of GSTP1 and cellular toxicity, independent of drug metabolism. Interestingly, there appears to be overlap between the catalytic and non-catalytic roles of GSTP1 protein relating to mitochondrial function, oxidative stress and MAP kinase regulation *in vivo*, highlighting a diverse set of pathways by which GSTP1 can operate. However, these functions are limited to only a few specific stress conditions and so further work is required to assess the application of these mechanisms to other stressors and disease states such as cancer, where GSTP1 over-expression is often associated. The use of hepatocytes derived from transgenic mice would provide a powerful tool in examining further roles of GSTP1 in applying novel *in vitro* assays to address *in vivo* functions. The limitations of the *in vitro* culture system used in this thesis are related to tumour and species type, therefore a universal function of GSTP1 may not be evident from the data presented. Despite this, a lack of conformity with a number of publications relating to GSTP1 function highlights the need to address cellular mechanisms other than those commonly associated with GSTP1 such as kinase regulation. There is much evidence provided in this thesis to suggest GSTP1 can mediate glutathione homeostasis although the extent to which GSTP1 is active in sulfhydryl homeostasis is unclear. The use of

novel *in vitro* assays has developed our understanding of the pathways mediated by GSTP1 and the effect, either direct or indirect, this has on cell regulation. Lastly, the data presented in this thesis suggest a novel role for GSTP1 in cytotoxicity. The use of a non-catalytic GSTP1 mouse provides an exciting *in vivo* model to further the understanding of this protein in cellular defence and provides an innovative system for investigating cellular regulation by GSTP1 in the aetiology of disease and toxicity.

8. Appendices

Target	Dilution	Clonality	Source
Actin	1:1000	Rabbit polyclonal	Sigma
ETF alpha	1:1000	Mouse monoclonal	Abcam
GAPDH	1:5000	Rabbit polyclonal	Sigma
GFP	1:2000	Rabbit polyclonal	Santa-Cruz
GSTA1	1:1000	Rabbit polyclonal	Prof. John Hayes (University of Dundee)
GSTM1	1:1000	Rabbit polyclonal	Prof. John Hayes (University of Dundee)
GSTP1	1:2000-1:5000	Rabbit polyclonal	Dr Kenny Ritchie (John Moore University, Liverpool)
HO-1	1:1000	Rabbit polyclonal	Abcam
LDH	1:1000	Goat polyclonal	Abcam
MnSOD	1:1000	Rabbit polyclonal	Novus Biologicals
NQO1	1:1000	Goat polyclonal	Abcam
p21	1:1000	Rabbit polyclonal	Santa-Cruz
p53 (DO-1)	1:1000	Mouse monoclonal	Santa-Cruz
p-ERK/ERK	1:1000	Rabbit polyclonal	Cell signalling
p-JNK/JNK	1:1000	Rabbit polyclonal	Cell signalling
p-Jun	1:1000	Rabbit polyclonal	Cell signalling
PrdxVI	1:1000	Rabbit polyclonal	Cell signalling
Streptavidin-HRP	1:1000-1:2000	Rabbit polyclonal	Cell signalling

Appendices I. A description of the antibodies used in this thesis.

Genes upregulated in GSTP shRNA HCT116 cells			**Genes downregulated in GSTP shRNA HCT116 cells**		
Gene	Name	Fold difference	Gene	Name	Fold difference
TSC22D1	TSC22 domain family	1.2 to 1.6	GSTP1	Glutathione S-transferase Pi	-9.2 to -13.7
MIR1978	microRNA 1978	1.4 to 1.5	MKNK2	MAP kinase interacting serine/threonine kinase 2	-1.4 to -2.5
RGS2	Regulator of G-protein Signalling 2	1.5 to 4.4	PTK2	Protein tyrosine kinase 2	-1.3 to -2.1
SULT1A1	Sulfotransferase 1A1	1.4 to 1.9	KLF2	Kruppel-like factor 2	-1.3 to -2.1
SULT1A4	Sulfotransferase 1A4	1.3 to 1.8	NP	Nucleoside phosphorylase	-1.5 to -2.2
S100A16	S100 calcium binding protein A16	1.3 to 2.6	ATP6V1B2	ATPase, H+ transporting, lysosomal V1 subunit B2	-1.4 to -1.9
TDP1	Tyrosyl-DNA phosphodiesterase 1	1.4 to 1.7	CYP24A1	Cytochrome P450 24A1	-1.3 to -4.7
FXR1	Fragile X Mental Retardation	1.4 to 5.0	TM4SF18	Transmembrane 4 L six family member 18	-1.2 to -1.9
ZDHHC6	Zinc finger, DHHC-type containing 6	1.3 to 1.5	ACAT1	Acetyl-Coenzyme A acetyltransferase 1	-1.4 to -1.7
NQO1	NAD(P)H dehydrogenase, quinone 1	1.2 to 1.8	AP3S1	Adaptor-related protein complex 3,	-1.3 to -2.1

				sigma 1	
ITGAE	Integrin, alpha E	1.2 to 1.4	TM9SF4	Transmembrane 9 superfamily protein member 4	-1.4 to -1.6
MGST2	Microsomal glutathione S-transferase 2	1.2 to 1.4	KLF6	Kruppel-like factor 6	-1.2 to -2.0
NSMCE2	MMS21 homolog	1.2 to 1.6	BOP1	Block of proliferation 1	-1.3 to -1.5
PTS	6-pyruvoyltetrahydropterin synthase	1.2 to 1.3	ZMYM6	Zinc finger, MYM-type 6	-1.2 to -1.5
MGC39900	hypothetical protein MGC39900	1.3	AP1M1	Adaptor-related protein complex 1, Mu1	-1.4 to -1.6
DBI	Diazepam binding inhibitor	1.3	STK39	Serine threonine kinase 39	-1.4 to -1.5
BCYRN1	Brain cytoplasmic RNA 1	1.3 to 1.6	PTPN1	Protein tyrosine phosphatase, non-receptor type 1	-1.4 to -2.0
TSTD1	Thiosulfate sulfurtransferase-like domain 1	1.2 to 1.4	PLAU	Plasminogen activator, urokinase	-1.5 to -1.6
ZMAT3	Zinc finger, matrin type 3	1.3 to 1.5	ANXA3	Aannexin A3	-1.2 to -1.6
PANK1	Pantothenate kinase 1	1.3 to 1.4	CARM1	Coactivator-associated arginine methyltransferase 1	-1.2 to -1.4
ALDH1L1	Aldehyde dehydrogenase 1	1.3 to 1.9	BMP4	Bone morphogenetic protein 4	-1.2 to -1.8

RBM47	RNA binding motif protein 47	1.2 to 1.3	BCL2L1	BCL2-like 1nuclear gene encoding mitochondrial protein	-1.4 to -1.5
TGIF1	TGFB-induced factor homeobox 1	1.3 to 1.4	DUSP5	Dual specificity phosphatase 5	-1.4
FADS1	Fatty acid desaturase 1	1.2 to 1.4	CDT1	Chromatin licensing and DNA replication factor 1	-1.4
S100A13	S100 calcium binding protein A13	1.2 to 1.4	GNPDA1	Glucosamine-6-phosphate deaminase 1	-1.3 to -1.6
PIR	Pirin (iron-binding nuclear protein)	1.2 to 1.3	PTPRA	Protein tyrosine phosphatase, receptor type, A	-1.2 to -1.4
GCNT3	Glucosaminyl (N-acetyl) transferase 3	1.2 to 1.4	SLC1A3	Solute carrier family 1	-1.3 to -1.9
OCIAD1	OCIA domain containing 1	1.2 to 1.3	VIL2	Villin 2 (ezrin)	-1.2 to -1.7
NR2F2	Nuclear receptor subfamily 2, group F, member 2	1.2 to 1.3	IDH3B	Isocitrate dehydrogenase 3 (NAD+) beta	-1.2 to -1.3
GRINA	Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1	1.2 to 1.3	C14orf173	Chromosome 14 open reading frame 173	-1.3
ZCCHC14	Zinc finger, CCHC domain containing 14	1.2	GMCL1	Germ cell-less homolog 1	-1.3
HYAL1	Hyaluronoglucosaminidase 1	1.2 to 1.4	C1orf85	Chromosome 1 open reading frame	-1.2 to -1.3

				85	
			CCDC21	Coiled-coil domain containing 21	-1.2 to -1.3
			KIAA0194	KIAA0194 protein	-1.2 to -1.3
			CMTM7	KLF-like MARVEL transmembrane domain containing 7	-1.2 to -1.4
			PES1	Pescadillo homolog 1	-1.2 to -1.3
			SPRY1	Sprouty homolog 1, antagonist of FGF signaling	-1.2
			CSNK1E	Casein kinase 1	-1.2 to -1.3

Appendices II. Gene expression data of mRNA profiles from GSTP1 silenced HCT116 cells.

mRNA expression profiles from 2 individual HCT116^{shGSTP1} clones were compared against mRNA expression profiles from HCT116 (untreated) and HCT116^{shcontrol} cells (n=3) in a four-way comparison as described in Figure 5.5. The profiles were collaborated and the table shows genes which were either upregulated or downregulated similarly across each set of comparisons. The values show the range in fold difference of gene expression across each comparison. Probes that exhibited an adjusted *P* value of <0.05 are expressed.

GSTP1 clone 1 vs untreated	***P* value**	**GSTP1 clone 2 vs untreated**	***P* value**	**GSTP1 clone 1 vs control plasmid**	***P* value**	**GSTP1 clone 2 vs control plasmid**	***P* value**
Translation - Translation in mitochondria	3.79E-05	Protein folding - Response to unfolded proteins	1.69E-07	Response to hypoxia and oxidative stress	1.06E-03*	Apoptosis - Endoplasmic reticulum stress pathway	4.55E-05
Protein folding - Folding in normal condition	6.02E-05	Protein folding - Folding in normal condition	3.11E-04	Immune response - Phagosome in antigen presentation	2.33E-02*	Immune response - Antigen presentation	1.25E-04
Immune response - Antigen presentation	2.62E-04	Response to hypoxia and oxidative stress	5.83E-04	Protein folding - Response to unfolded proteins	3.41E-02*	DNA damage - DBS repair	3.13E-04
Cell cycle - Mitosis	2.84E-04	Translation - Translation in mitochondria	1.49E-03	Apoptosis - Apoptotic mitochondria	4.46E-02*	Protein folding - ER and cytoplasm	1.01E-03
Protein folding - Response to unfolded proteins	3.05E-04	Protein folding - Protein folding nucleus	9.36E-03*			Signal transduction - Leptin signaling	1.29E-03
Immune response - Phagosome in antigen presentation	1.12E-03	Protein folding - ER and cytoplasm	1.27E-02*			Signal transduction - WNT signaling	1.54E-03
Inflammation - Protein C signaling	1.90E-03						
Response to hypoxia and oxidative stress	2.21E-03						

Transcription - mRNA processing	2.53E-03						

Appendices III. Enrichment analysis of cellular processes upregulated in HCT116 cells silenced for GSTP1.

The table shows statistically significant processes which are upregulated in HCT116^{shGSTP1} cells compared to HCT116 untreated or HCT116^{shcontrol} cells. * signifies processes which are not statistically significant but are common across other comparisons.

GSTP1 clone 1 vs untreated	*P* value	GSTP1 clone 2 vs untreated	*P* value	GSTP1 clone 1 vs control plasmid	*P* value	GSTP1 clone 2 vs control plasmid	*P* value
Cholesterol Biosynthesis	1.18E-05	Cholesterol Biosynthesis	1.33E-09	Peroxisomal branched chain fatty acid oxidation	5.68E-04	Apoptosis and survival_Endoplasmic reticulum stress response pathway	9.20E-07
Immune response_Neurotensin-induced activation of IL-8 in colonocytes	1.17E-04	Mechanisms of CFTR activation by S-nitrosoglutathione (normal and CF)	3.75E-06	Catecholamine metabolism / Human version	1.92E-03	Immune response_Oncostatin M signaling via MAPK in human cells	1.74E-04
Mechanisms of CFTR activation by S-nitrosoglutathione (normal and CF)	5.05E-04	CFTR folding and maturation (norm and CF)	6.90E-06	Triacylglycerol metabolism p.1	2.89E-03	Immune response_Antigen presentation by MHC class I	2.50E-04
CFTR folding and maturation (norm and CF)	8.09E-04	Apoptosis and survival_HTR1A signaling	7.39E-05	n-6 Polyunsaturated fatty acid biosynthesis	4.97E-03	Nicotine signaling in dopaminergic neurons, Pt. 2 - axon terminal	4.59E-04
Apoptosis and survival_HTR1A signaling	1.20E-03	Neurophysiological process_Dopamine D2 receptor transactivation of PDGFR in CNS	4.53E-04	n-3 Polyunsaturated fatty acid biosynthesis	4.97E-03	Immune response_Antigen presentation by MHC class II	4.66E-04
G-protein signaling_G-Protein alpha-q signaling cascades	2.46E-03	Catecholamine metabolism / Human version	4.53E-04			Development_Role of IL-8 in angiogenesis	5.81E-04
Cytoskeleton	2.46E-03					Apoptosis and	6.39E-04

remodeling_RalA regulation pathway						survival_Caspase cascade	
Catecholamine metabolism / Human version	2.86E-03					Triacylglycerol metabolism p.1	7.33E-04
NGF activation of NF-kB	3.04E-03						

Appendices IV. Enrichment analysis of cellular pathways upregulated in HCT116 cells silenced for GSTP1.

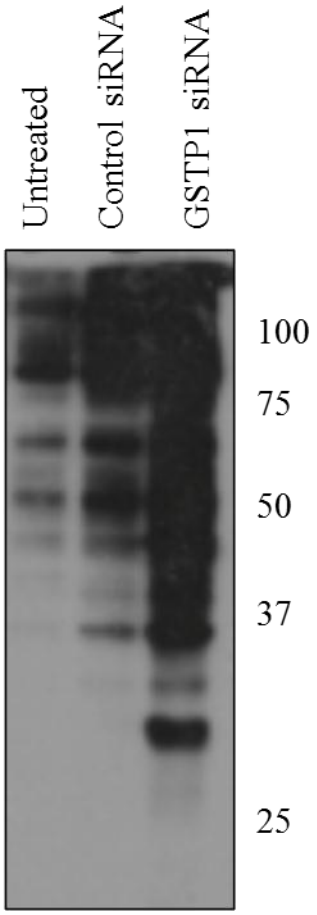
The table shows statistically significant pathways which are upregulated in $HCT116^{shGSTP1}$ cells compared to HCT116 untreated or $HCT116^{shcontrol}$ cells. * signifies pathways which are not statistically significant but are common across other comparisons.

GSTP1 clone 1 vs untreated	***P* value**	**GSTP1 clone 2 vs untreated**	***P* value**	**GSTP1 clone 1 vs control plasmid**	***P* value**	**GSTP1 clone 2 vs control plasmid**	***P* value**
Aminoacyl-tRNA biosynthesis in cytoplasm	3.54E-10	Aminoacyl-tRNA biosynthesis in cytoplasm	1.20E-05	Cytoskeleton remodeling_Cytoskeleton remodeling	4.88E-05	Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	1.59E-09
Transcription_Ligand-dependent activation of the ESR1/SP pathway	6.08E-04			Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	5.95E-05	Cytoskeleton remodeling_Cytoskeleton remodeling	5.28E-06
				Cell cycle_Cell cycle (generic schema)	1.56E-04	Cytoskeleton remodeling_Keratin filaments	5.50E-06
				Cell adhesion_Gap junctions	1.56E-04	Cell adhesion_Gap junctions	1.54E-05
				Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis	3.98E-04	Cytoskeleton remodeling_Neurofilaments	5.38E-05
				Translation_Translation regulation by Alpha-1 adrenergic receptors	5.02E-04	Cytoskeleton remodeling_Reverse signaling by ephrin B	1.94E-04
						Cell adhesion_Endothelial cell contacts by junctional mechanisms	6.98E-04

						Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity	
						Cell adhesion_Chemokines and adhesion	2.00E-03
						Cytoskeleton remodeling_Role of Activin A in cytoskeleton remodeling	2.11E-03

Appendices V. Enrichment analysis of cellular pathways downregulated in HCT116 cells silenced for GSTP1.

The table shows statistically significant pathways which are upregulated in HCT116^{shGSTP1} cells compared to HCT116 untreated or HCT116^{shcontrol} cells. * signifies pathways which are not statistically significant but are common across other comparisons.



Appendices VI. S-nitrosylated proteins in HCT116 cells silenced for GSTP1.

HCT116 cells were transiently transfected with 10nM siRNA targeted against GSTP1 or control siRNA and grown for 72 hours. Lysates were prepared from the cells and sulfhydryl groups blocked with S-Methyl methanethiosulfonate (MMTS). S-nitrosylated proteins were reduced with sodium ascorbate and labelled with Biotin-HPDP. Lysates (10µg) were resolved on a non-reducing SDS-PAGE gel and analysed by Western blotting. A streptavidin-HRP antibody (1:1000) was used for the detection of biotinylated proteins.

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